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Investigation of L-Carnitine and Cadmium Impact on mTOR pathway-related Genes Obtained from Rats' Liver

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Approval

We certify that we have read the thesis submitted by Duaa Abdulmohsin titled **"Investigation of L-Carnitine and Cadmium Impact on mTOR pathway-related genes Obtained from Rats Liver**" and that in our combined opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Educational Sciences.

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Declaration

I hereby declare that all information, documents, analysis, and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Duaa Abdulmohssin

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I dedicate this thesis to my parents, Mr. and Mrs. Abdulmohsin Alhaj. May Allah bless them.

Duaa Abdulmohssin

Abstract

Investigation of L-Carnitine and Cadmium Impact on mTOR Pathway-Related Genes Obtained from Rat' Liver

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Background: The liver is an essential organ that occupies the upper right quadrant of the abdominal cavity. It is responsible for removing toxins from the blood supply of the body, keeping glucose levels under control. The liver is a primary organ in charge of detoxifying and metabolizing a variety of substances that result in reactive oxygen species (ROS). The increased creation of ROS in the presence of liver illness may lead to increased levels of peroxidation of lipid membranes and the formation of inflammatory molecules, resulting in liver cell damage and death. Patients with liver disease may benefit from L-Carnitine by reducing oxidative stress, decreasing inflammation, and stimulating enzymes required to defend against oxidative damage. Another factor that contributes to liver disease is the toxicity caused by heavy metals, such as cadmium. It has been shown that cadmium increases ROS production in the body. Cadmium toxicity varies depending on the amount and duration of exposure; however, it is hypothesized to activate autophagic and apoptotic pathways through the formation of ROS. Furthermore, ROS causes transcriptional alterations in cellular proteins and impacts the mTOR network signaling. A broad range of internal and external events can cause the mTOR signaling pathway to regulate cell development. Therefore, the focus of this thesis was to investigate the expression of *mTOR*, *Akt1*, and *Akt2* in rat livers whose cadmium and L-Carnitine were administrated and to understand the mechanism of how cadmium and L-Carnitine interact with the mTOR pathway.

Materials and Methods: A total of 24 female rats were divided into four groups. The first group was administrated cadmium, the second group was administrated cadmium and L-Carnitine, the third group was administrated L-Carnitine, and the fourth group was the control group and they were not administrated any drugs. The rats were breaded in the Near East University Experimental Animal Facility, and they were dissected and the livers were stored in a -80° freezer. RNA was extracted followed by an RNA purity test using Nano-drop

spectrophotometer. cDNA was synthesized using the extracted RNA. Gene expression level in each sample was evaluated using real-time polymerase chain reaction (RT-PCR). Furthermore, a statistical analysis was performed on the RT-PCR data to evaluate the expression of the desired genes, and all the p values more than 0.05 were declared insignificant.

Results: When comparing the drug-administered rat liver groups to the control group, the results indicated that there was no statistically significant variation in gene expression levels.

Conclusion: In this study, the expression of genes associated with the mTOR signaling pathway was evaluated. This research focused on two drugs since previous studies have demonstrated that these two drugs have effects on both animals and humans. No studies have been conducted on rat livers to determine the involvement of cadmium and L-Carnitine in the genes associated with the mTOR pathway.

Keywords: Liver, L-Carnitine, Cadmium, mTOR pathway

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List of Abbreviations

AIF:	Apoptosis-inducing factor
ATP:	Adenosine triphosphate
cDNA:	Complementary Deoxyribonucleic acid
CICs:	Circulating inflammatory cells
CT:	Cycle threshold
DMT1:	Insulin-like growth factors 1
IGF1:	Insulin-like growth factors 1
IL:	Interleukin
IRE:	Iron representative element
IRP:	Iron representative protein
MDA:	Melanodialdehyde
MT:	Metallothionein
mTOR:	Mammalian target of rapamycin
NASH:	Non-alcoholic steatohepatitis
PPM:	Part per million
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species

CHAPTER I

Introduction

The following chapter is a general overview of the drugs used in the study. The chapter explains the aspects of the drugs, including the background, applications, functions, and side effects. This chapter also involves the aspects of the organ system, liver, and mTOR pathway, due to the fact that these variables are the base of this research.

1.1 Liver Function

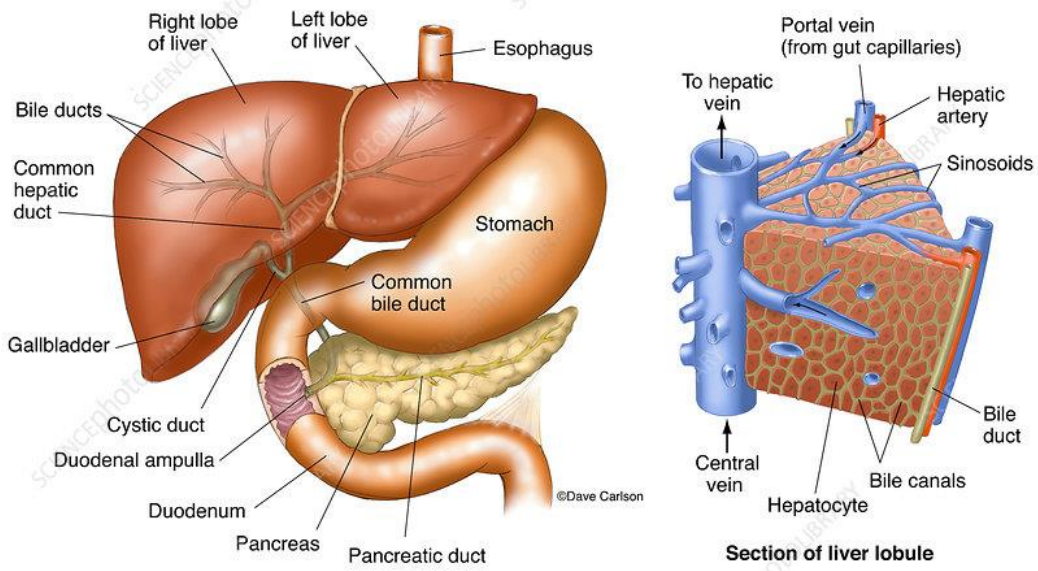
Since the dawn of medicine, the liver has piqued the interest of humankind. The architecture, structure, and functionality of the liver, on the other hand, have transformed radically during the previous 1800 years (Leiskau and Baumann, 2017). The liver occupies the upper right quadrant of the abdominal cavity. At any time, the liver retains around one pint (13%) of the blood supply of the human body (Abdel-Misih & Bloomston, 2010). The liver has two divisions of lobes, and both have eight segments with 1,000 lobules each (small lobes). Small ducts (tubes) link these lobules to create the common hepatic duct, connecting to more giant ducts (Figure1.1) (McINDOE, 1927). When liver cells produce the bile, it is transported to the gallbladder and duodenum (the first section of the small intestine) through the common hepatic duct (Shiojiri, 1997). The liver integrates the metabolic activity of the body *via* glucose metabolism and fatty acid oxidation; in addition, it is essential for maintaining blood glucose homeostasis. As well as two-thirds of the oral glucose cytoplasm burden is disposed of by the liver (Leiskau and Baumann, 2017).

Furthermore, the liver highly influences endocrine regulation, and it generates most of the circulating mitosis-inducing (mitogenic) polypeptide hormones insulin-like growth factors 1 and 2 (IGF1 and IGF2) in response to pituitary growth hormone activation, which has anabolic and metabolic effects, as well as regulating cellular proliferation and is also essential for growth and development (Pierzchała et al., 2012). Another critical role of the liver is storing and releasing large amounts of whole blood and plasma, influencing the circulating blood volume. However, the liver is the primary site of drug metabolism in the body since most xenobiotics are absorbed by the gastrointestinal tract and move into the portal vein to the liver. Most medicines are delivered into hepatocytes

by ATP-dependent solute carriers and processed in the smooth endoplasmic reticulum of the hepatocytes (Remmer, 1970).

Figure 1.1

The anatomy of the liver (Hopkinsmedicine., 2021).



1.2 L-Carnitine

Carnitine ($C_7H_{15}NO_3$) is a dipolar amino acid-like molecule that occurs naturally and extensively spreads throughout nature (Bieber, 1988). It is exceptionally well-preserved in organs such as skeletal muscles, heart, and brain in the human body. Carnitine has many physical characteristics, including water solubility, and it is a whitish crystalline powder. It has a molecular mass of 161.2 g per molecule and a melting point of 210-212°C (Dayanand, C. D., et al., 2011). In terms of UV absorption, carnitine is less effective than its natural degradant crotonyl betaine, which has a many-fold greater capacity attributed to two conjugated double bonds (D'Acquarica et al., 1999; Dayanand, C. D., et al., 2011). The essential amino acids lysine, the co-factor L-ascorbic acid, and methionine are necessary for carnitine biosynthesis that transpires mainly in the liver and kidney (Bremer, 1983). Moreover, for carnitine to have a biological impact, it must be combined with high-quality proteins or whole proteins having a rich biological value in the diet (Steiber et al, 2004).

Carnitine originates as two kinds of stereoisomers, the most common of which is L-Carnitine, which is naturally occurring and physiologically active. The least common is D-Carnitine, which is determined to be biologically inactive. On the other hand, D-Carnitine is utilized to decrease the L-Carnitine levels in the tissues to a certain extent (Spasov, A. A., et al., 2006).

L-Carnitine is a critical component of the mitochondrial matrix's metabolic process of fatty acid oxidation, which generates energy. It transfers activated fatty acids (acyl CoA) from the cytoplasm through the inner mitochondrial membrane (Walter, 1996). For the most part, carnitine transport is required to transfer fatty acids with more than fourteen carbon atoms, which may come from dietary sources or fats released from adipose tissue (Savic et al., 2020). However, in the case of L-Carnitine deficit, it is indicated by low carnitine in the blood and tissues compared to the average level. L-Carnitine deficiency occurs as a result of a variety of factors. Therefore, decreased intake of food that is rich with L-Carnitine such as dairy products and meat, reduced *in vivo* synthesis of carnitine as a result of liver malfunction, excessive loss through urine while undergoing diuresis, hemodialysis, or diarrhea, and increased loss through sweating (Cruciani et al., 2006).

1.2.1 *Applications and Benefits of L-Carnitine*

L-Carnitine performs a wide variety of roles in the body. One of the main functions is that L-Carnitine plays a catalytic action in the mitochondrial burning of fatty acids and is just required in modest quantities; it is not consumed and is accessible for use repeatedly throughout the course of the activity (Harmeyer, 2002). The other primary function relies on the metabolic role of L-Carnitine as a buffer for excessive acyl residues (Pekala et al., 2011). Free L-Carnitine is transformed to L-Carnitine ester (mostly acetyl L-Carnitine) during the conversion process, where it requires significant amounts, and the consumption will be nearly entirely (Adeva-Andany et al., 2017).

There has been broad support for carnitine therapy in a wide range of inherited and acquired diseases, both to restore low L-Carnitine levels in complications associated with defect states and to remove toxic metabolites even when plasma and tissue L-Carnitine quantities are within a normal range (Kraemer et al., 2008). Treatments are available in

both oral and intravenous forms. While L-Carnitine uses are many, the rarity of most particular conditions for which it is prescribed means that reports of its effectiveness have mostly been anecdotal. There are few controlled studies to support these claims. Furthermore, without proper neutralization, free radicals created by the body eventually produce oxidative damage to the cell membrane and organelles, which may be fatal if not addressed (Sailaja Rao et al., 2011).

The ability of the body to quench oxidants is reliant on the antioxidant capacity of the cells (Pham-Huy et al., 2008). If the amount of antioxidant in the diet does not satisfy the needs for maintaining a proper antioxidant status, oxidative stress may occur (Jacob & Burri, 1996). L-Carnitine is thought to have antioxidant qualities because it acts as a metal chelator, which reduces the number of unbound iron ions in the body (Gülçin, 2006). Several radicals, including superoxide, hydroxyl radicals, and hydrogen peroxide, are neutralized in the existence of L-Carnitine in laboratory studies (Kocer et al., 2007).

When it comes to detoxification and metabolism of numerous substances that form ROS, the liver is the primary organ to check for ROS. The increased creation of ROS in the presence of liver illness may lead to increased peroxidation of lipid membranes and the formation of inflammatory molecules, which may result in damage and cell death in the liver cells themselves (Muriel, 2009). The injury or death of hepatocytes causes an increase in the secretion of liver enzymes in the bloodstream (Contreras-Zentella & Hernández-Muñoz, 2016). Patients suffering from chronic liver illness, particularly liver cirrhosis, are often found to be suffering from secondary carnitine insufficiency (Sato et al., 2020). The liver is a primary location for carnitine synthesis, illness in the liver may affect carnitine synthesis, hastening the development of the disease. According to the findings of a recent meta-analysis, L-Carnitine supplementation enhances liver function in patients that suffer from non-alcoholic steatohepatitis (NASH) by decreasing histological steatosis and the number of NAS tests that are necessary. (Li & Zhao, 2021).

Moreover, Okabayashi and colleagues (2020) found that L-Carnitine might enhance postoperative liver function in individuals who had been surgically hepatectomized (Okabayashi et al., 2020). L-Carnitine has been shown to attenuate the inflammatory response because of the transfer of -oxidized long-chain fatty acids into mitochondria and

the excretion of harmful chemicals during fatty acid metabolism (Kraemer et al., 2008). It also serves as a free radical scavenger by lowering ROS generation. In this way, L-Carnitine may benefit patients with liver disease by reducing oxidative stress, decreasing inflammation, and stimulating enzymes required to defend against oxidative damage. High serum liver enzymes in patients with liver disease may be lessened as a result.

L-Carnitine reduced Malondialdehyde (MDA) in broiler contents when augmented into their diet. In a similar situation, the antioxidative capability of liver broilers increased when treated with L-Carnitine (Wang et al., 2013). L-Carnitine also has the additional benefit of improving the antioxidant state of chickens by stimulating the production of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Rehman et al., 2017). Among its many functions, L-Carnitine is capable of suppressing microsomal peroxidation, promoting oxygen consumption, and keeping the number of fatty acids in the cell at a bare minimum (Dayanandan et al., 2001).

Moreover, when L-Carnitine is supplemented into broiler feed, it has been shown to lower a variety of blood metabolites, including liver enzymes, cholesterol, triglycerides, and low-density lipoprotein. L-Carnitine is well known for enhancing broiler diets resulting in lower blood cholesterol levels. There is a possibility that the decrease in serum cholesterol is linked to the stimulatory effect of L-Carnitine on lipid oxidation and metabolism. L-Carnitine's stimulatory effect on lipid oxidation and metabolism might explain the decline in blood cholesterol levels. L-Carnitine supplementation also reduced the cholesterol and triglycerides levels found in egg yolks (Parizadian et al., 2011). On the other hand, other studies did not find a statistically significant impact on serum cholesterol. The decrease in serum triglycerides is most likely owing to the enhanced transport of fatty acids into the mitochondria for oxidation that was detected. L-Carnitine stimulates lipase activity while inhibiting lipoprotein lipase activity, consequently, triglycerides are converted into fatty acids and glycerol in the body (Liu et al., 2020). Poultry immunity is composed of both cell-mediated and humoral components. Several studies have shown that supplementing birds with L-Carnitine increased their humoral immune response against several viral illnesses such as Newcastle disease, avian influenza, and ascites (Rehman et al., 2017). One of the most often reported explanations

for the therapeutic benefits of L-Carnitine is its ability to prevent immune cells apoptosis (Vescovo et al., 2002). The immunological regulating effect of L-Carnitine, according to some experts, is attributed to the antioxidant activity of L-Carnitine. Several studies have revealed that endogenous L-Carnitine is a significant predictor of better immunological and inflammatory responses to invading pathogens (Rehman et al., 2017).

1.2.2 *Side Effects of L-Carnitine*

Supplement-associated side effects in human clinical trials of oral L-Carnitine supplementation are documented in reviews and meta-analyses. The most common side effects are nausea and vomiting occasionally, diarrhea, and an unpleasant odor in the urine and sweat (Goa & Brogden, 1987). Increasing levels of trimethylamine N-oxide, which are linked to L-Carnitine supplementation, has been hypothesized to have potentially adverse consequences in the long run. This is because gut bacteria degrade L-Carnitine into trimethylamine, which is then absorbed by the gut and transformed by the liver into Trimethylamine N-oxide (Fukami et al., 2015).

Similar to L-Carnitine, Trimethylamine N-oxide may also be found in naturally occurring amounts in animal products, such as meat. Trimethylamine N-oxide is implicated in the development of atherosclerosis, renal disease, and diabetes in rat and cell studies; in line with this, atherosclerosis was found to deteriorate in a mouse model of atherosclerosis when L-Carnitine is supplemented (Koeth et al., 2019). Many clinical studies in persons at risk for cardiovascular disease have shown an elevated risk of mortality in the presence of escalating blood content of trimethylamine N-oxide of these individuals. High blood L-Carnitine concentrations are shown to be connected with an increased risk of cardiovascular disease in a single clinical investigation (Dambrova & Liepinsh, 2015).

1.3 Cadmium

Cadmium, a naturally occurring metallic element, is found in the crust of earth and seas and the surroundings in minimal concentrations and tad amounts in the human body. Cadmium is predominantly identified as a malleable metal with a silver-white or bluish-white shade; weighs 112.41 gram per molecule of molecular mass. Due to its rarity, cadmium may be found in just a few locations in the universe, yet its abundance can rise

regionally to levels as high as one part per billion or more in certain rocks, depending on their composition. (Morrow, 2010). Cadmium is a very toxic heavy metal that is a byproduct of the metallurgy of zinc, lead, and copper, and cannot be eliminated from the process. The crustal abundance of Cadmium has a varying concentration ranging from 0.1 to 0.5 parts per million (PPM), although relying on many other variables, a spectrum of values has also been observed (Viets, 1978).

The chemical characteristics of cadmium are similar to zinc in general, although they are more pronounced in reducing circumstances and when combined with other metals. Cadmium may act similarly to calcium in oxides, fluorides, and carbonates, as well as in oxidizing environments, a comparatively significant number of complex ions with other ligand species, such as ammonia, cyanide, and chloride, are also formed. Furthermore, cadmium is a very reactive metal; therefore, the usage of this heavy metal is limited in most applications. When mixed with weak hydrochloric or sulfuric acids, it dissolves slowly, but it dissolves faster when combined with hot dilute nitric acid (Morrow, 2010).

On the other hand, when exposed to the elements in an environment, cadmium forms a protective oxide covering, similar to that formed by zinc, which reduces the rate of corrosion and oxidation (Kautek, 1988). Both metals have low corrosion rates when exposed to pH values ranging from 5 to 10, but their corrosion rates increase significantly when exposed to more acidic and alkaline circumstances. In marine or alkaline environments, cadmium is typically used, however, zinc is frequently just as good in substantial industrial exposures involving sulfur or ammonia (Hayat et al., 2019). When zinc sulfide ore concentrations are beneficiated, smelted, and refined for use in other products, the metal cadmium is produced as a by-product. A small amount is created as a derivative of processing complicated zinc, lead, and copper ores. Recycling abandoned cadmium goods and cadmium-containing industrial wastes, on the other hand, provides for a major portion of current cadmium productivity (Sadegh Safarzadeh et al., 2007).

1.3.1 *Applications and benefits of Cadmium*

The unique chemical and physical features of cadmium make it very easy to be utilized in multiple modern technologies. Cadmium-coating *via* electroplating is common in industrial applications, particularly in the aerospace sector, to prevent and control other alloys against corrosion (Mohan Kumar et al., 2017). Furthermore, many alloys have cadmium as a component. Antifriction, soldering, and electrical materials all benefit from their improved mechanical and thermal qualities (Carr, 2000).

Cadmium's huge neutron sectional cross-section makes it an outstanding blockade for controlling nuclear fission (Martelli et al., 2006). Moreover, with Ni-Cd batteries, the Cd/Cd2+ redox couple (–0.4V) power the anode. Using this energy storage technology is beneficial, and it is now successfully included in many applications (Rydh & Karlström, 2002).

Among their numerous applications as pigments, chalcogenides, which is a class of cadmium compounds, interact with light and display optical properties that make them ideal for use in plastics, paints, enamels, and inks. (Cole & le Brocq, 2007). When cadmium chalcogenides release light, it is regularly employed in electronics and various display devices, among other applications (Jin & Zhai, 2020). Cadmium chalcogenides crystals with precisely calibrated nanometric dimensions may now be synthesized under strict regulations, according to research published in the last several years. Quantum confinement allows nanocrystals, commonly referred to as "quantum dots," to have size-dependent tunable emission spectra, opening up a broad array of potential (Rzigalinski & Strobl, 2009). These are being closely scrutinized all around the globe, and when particular difficulties, such as toxicity, are adequately solved, they might result in significant industrial advancements in the future.

1.3.2 Side Effects of Cadmium

The primary sources of occupational exposure to cadmium-containing products are the manufacturing and disposal of these compounds. Phenomena like wind-driven suspension of cadmium particles near the Earth's outer shell or volcanic gas dispersion are examples of non-human causes of air pollution (Hutton, 1983).

Fertilizers and other agricultural practices facilitate the movement of cadmium from soil and minerals, hence increasing the metal's concentration in food and water (Chen et al., 2007).

Accordingly, cadmium's primary routes of exposure are inhalation and ingestion. Cigarette smokers absorb cadmium in unusually high concentrations (Sharma, Kjellström, and McKenzie, 1983). The respiratory system is very vulnerable to the effects of cadmium from contaminated environments. Therefore, intensive intake of cadmium dust and vapor targets the kidney, colon, and liver; inducing significant tubular damage due to this accumulation. Once absorbed, cadmium half-life in humans is exceptionally prolonged, with estimates ranging from more than 20 years to 100 years. The long residence period in the bloodstream and urine shows that Cadmium may be effectively retained in the body and then excreted via detoxification (Genchi et al., 2020). Different investigations have demonstrated the presence of Cd-Se linkages in rat plasma and mixed minerals of calcium-cadmium in the kidneys of marine animals (Stajn et al., 1997). The findings are possibly attributable to the variety of the analyzed samples and the analytical procedures used implying that cadmium transfers among various biomolecules in mammalian tissues (Moulis et al., 2010).

The clinical symptoms that humans experience due to cadmium exposure are closely related to the distribution of the metal throughout the body (Bernhoft, 2013). Inhalation creates respiratory stress and may damage the respiratory system. High cadmium concentrations in contaminated air are associated with the development of emphysema, anosmia, and chronic rhinitis. Cadmium is designated to be a human pulmonary carcinogen due to the high prevalence of lung cancer among workers exposed to this metal in their jobs (Rafati Rahimzadeh et al., 2017).

Cadmium may cause or contribute to the development or progression of other types of cancers. However, due to a paucity of evidence, the spectrum of human cadmium carcinogenicity has not been expanded beyond lung carcinoma to include different types of cancer (Liu et al., 2009). Cadmium overdose has been linked to anemia and eosinophilia, while cadmium buildup in the kidneys results in

nephropathy and proteinuria (Sonone, Swaroop S, et al., 2020). Osteomalacia and osteoporosis are two conditions that may occur due to cadmium interfering with the development and maintenance of bones (Kazantzis, 2004).

In case of inhalation, cadmium dust is often formed of cadmium oxides or cadmium dichloride, depending on their composition. Cadmium particles pass *via* primary olfactory neurons to their terminals in the olfactory bulb, where they get removed. The migration of certain other metals into the brain is more extensive, such as manganese, as opposed to cadmium, which tends to collect in the olfactory bulb (Zayed & Philippe, 2009). The lungs, for example, are another location of cadmium buildup after inhalation, as in smokers. Despite the lung epithelium's effectiveness as a barrier to hazardous substances and heavy metals, cadmium may nevertheless penetrate through alveolar cells and reach the bloodstream. (Foulkes, 2000). The exact method through which Cadmium enters the circulation is still a mystery to scientists. Cadmium is likely carried through the body through transporters or pathways that are specific to other ions and biomolecules, whether it is a free cation or coupled to chelators like glutathione or cysteine.

Similarly, the intake of cadmium-contaminated food and water is another significant form of exposure. The acidic medium of the digestive system facilitates cadmium transportation, which is mediated by vast specificity proton-metal co-transporter DMT1, located within the enterocytes apical membrane. In the human enterocyte model, DMT1 suppression reduces cadmium transportation, while overexpression significantly elevates cadmium absorption. When rats are on an iron-deficient diet, they acquire vast quantities of cadmium, which is considerably reduced when the diet is supplemented with more iron (Sarkar et al., 2013). The absorption of cadmium gets enhanced in iron-deficient environments. Due to the presence of an iron-responsive element (IRE) in DMT1, a splicing variant of DMT1 that is localized in the plasma membrane is produced by duodenal enterocytes. Additionally, regulatory proteins (IRP) boost the translation. In the non-coding regions of controlled mRNA, the translational regulators IRP1 and IRP2 interact with the intergenic region (IRE) (Tallkvist et al., 2001). Toxic effects from ingesting cadmium

are thus closely linked to an organism's total iron level. As a result, DMT1 transport of cadmium permits the toxic metal to reach the intestinal epithelium, particularly under iron-deficient conditions (Martelli et al., 2006).

When exposed to excessive amounts of cadmium, necrosis and apoptosis occur in cells. The dosage and the kind of cell that is being targeted determine the proportion of cell death that occurs. (Habeebu et al., 1998). Cadmium's pro-apoptotic effect is controlled by a variety of signaling pathways, which may lead to caspase-dependent or caspase-independent cell death. Apoptosis-inducing factor (AIF) and endonuclease G proteins are translocated within the nucleus as a result of the latter. In both forms of apoptosis processes, the increase in intracellular calcium appears to be a significant factor (G. Liu et al., 2016). However, an intracellular calcium imbalance may be responsible for the cascade of apoptotic events triggered by cadmium (Zhou et al., 2015).

Cadmium's hepatotoxicity may also be linked to inflammation, as shown by the fact that polymorphonuclear neutrophils (PMN), as well as Kupffer cells, enter the injured liver after severe exposure, contributing to the hepatotoxicity by boosting the inflammatory mediators and stimulating necrosis (Iimuro et al., 1997). Preliminary studies show that activated Kupffer cells, which are responsible for the release of inflammation mediators, enhance the expression of adhesion molecules, which in turn leads to inflammation and secondary liver damage following cadmium-induced hepatotoxicity. Cadmium's hepatotoxic effects are greatly reduced if Kupffer cells are deliberately destroyed, inhibited, or silenced. Even though the precise mechanism by which activated Kupffer cells contribute to cadmium liver damage is unknown, it is known that when exposed to the metal, ROS, nitric oxide, and cytokines are released by activated Kupffer cells, which may affect hepatocytes directly (Rikans & Yamano, 2000). *Interleukin*-1 (*IL*-1), *Interleukin*-6 (*IL*-6), and *Interleukin*-8 (*IL*-8) gene expression in human hepatoma cell line HepG2 were suppressed by cadmium-induced acute-phase protein production and anti-*TNF* antibodies. Hepatocytes or HepG2 cells exposed to cadmium have also shown increased expression of *IL*-1beta, *TNF*-alpha, *IL*-6, and *IL*-8 (Låg et al., 2010).

Cell adhesion molecule-1 I(MAC-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, P-selectin, and 2-integrin Mac-1 are also produced in the liver by *TNF*-a and *IL-1*a. These adhesion molecules have been shown to promote the recruitment, adhesion, and activation of circulating inflammatory cells (CICs) (Jaeschke et al., 1996). Hepatic endothelial cells may be the main target of damage in the liver caused by cadmium (Rikans and Yamano, 2000). This local ischemia in the parenchyma is indicative of cadmium-induced hepatic endothelial degeneration, as seen by the ejection of damaged cells into the capillary lumen. The luminal surfaces of the endothelial cells were substantially damaged, resulting in considerable loss of fenestrations, after cadmium (3 mg/kg CdCl2) injection into rats. Disruption to the hepatic endothelial cells was detected within three hours of treatment. Endothelial cells that have been destroyed block the capillary lumen, causing ischemia in the affected area. Activating Kupffer cells, the release of inflammatory mediators, and the stimulating inflammatory cells, primarily leukocytes and PMN, may then occur as a consequence of this (Kuester et al., 2002).

Inflammatory cells clump together in sinusoids and adhere to endothelial cells in the liver, resulting in inflammation. The last phase in Cadmium hepatotoxicity is transmigration, that is regulated by platelet–endothelial adhesion molecule-1 (PECAM-1), indicating that adhesion molecules such as E-selectin, Mac-1, and ICAM-1 play an important role throughout this process (Sørensen et al., 2015).

1.4 mTOR Pathway

The target of rapamycin (TOR) is a protein kinase that has been highly conserved throughout evolution. When mTOR interacts with other proteins, it forms two complexes, mTOR complexes 1 and 2 (mTORC1 and mTORC2), which regulate cell growth and proliferation (Zarogoulidis et al., 2014). mTORC1 is linked to Raptor, that attaches to proteins that are mTORC1's direct substrates, while mTORC2 is linked to Rictor, that attaches to proteins that are mTORC2's indirect substrates (Feldman et al., 2009). In contrast to signaling via mTORC2, signaling through mTORC1 is considerably well known. mTORC1 is a critical node in cellular regulation that influences cell proliferation (Toschi et al., 2009).

PI3K/AKT/mTOR signaling can be triggered by various molecules, including insulin, glucose, and a variety of growth hormones and cytokines (Bodine, 2006). Typically, these molecules activate receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), which in turn activate PI3K, resulting in the production of phospholipids. These signals cause the activation of PI3K downstream effectors such as AKT and the mTORC1. PI3K signaling is dependent on protein kinase B (AKT) activity. mTORC2, which interacts with AKT's regulatory hydrophobic region to phosphorylate it at Ser473, is responsible for mediating the activation of the AKT signaling pathway. Activated AKT is translocated into different cell compartments, where it activates a variety of downstream substrates, such as small G protein regulators, metabolic enzymes, transcription factors, cell cycle regulators, and transcription factors (Sun et al., 2020). mTORC1 is an essential downstream branch of AKT. At Ser 2448, MTORC1 may either be activated directly or indirectly by AKT, which has been linked to *Tuberous Sclerosis Complex* 2 (*TSC2*) via phosphorylation of *TSC2* (Wang & Proud, 2006).

Oxidative stress is generated when the mTOR system controls cell growth in response to various internal and external triggers. mTOR has two distinct roles when it comes to stress monitoring. ROS causes transcriptional alterations in cellular proteins and impacts the mTOR network signaling (Chatterjee et al., 2016). On the other hand, ROS suppresses mTORC1 through phosphorylating *TSC2* and activating AMPK. When ROS are generated, AMPK is activated, phosphorylates, and activates *TSC2*, which results in mTORC1 inhibitory activity (Li et al., 2010). However, autophagy may be sped up by oxidative stress, which causes the mTORC1 to take on additional roles. the activity of autophagy-related proteins (ATG) is inhibited by mTORC1 in the complex formed by the autophagy-related proteins (ATG) ATG 1, ATG13, and ATG101. Stress may be used to reactivate mTORC1 and, as a result, destroy the mTORC1 signaling pathway (W. Wang et al., 2018). Autophagic lysosome reformation occurs when ATG1 is released from the ATG protein complex and promotes the formation of protolysosomal extension, which is defined by the presence of lysosomal-associated membrane protein 1 (LAMP1) and microtubule-associated protein 1A/1B light chain 3 (LC3) from the autophagolysosome (Lian et al., 2020).

Toxicity may trigger pathways in cells that behave as death or survival indicators. Signaling systems within every organ determine their vital thresholds. Toxins may cause cells to utilize a controlled, pre-established signaling route to maintain the organ's normal homeostasis in order to maintain its normal function (Orrenius et al., 2011). For the most part, hazardous metals cause oxidative stress as the first response. From there, many signaling pathways work in concert to protect the integrity of cellular and molecular homeostasis. Free oxygen or hydroxyl radicals may be generated in cells when hazardous metals combine with lipid membranes, which are particularly reactive. (Valko et al., 2005).

Due to the extended half-life of cadmium, the harmful effects are magnified and are linked to oxidative phosphorylation inhibition, glutathione depletion, suppression of antioxidant enzyme activity, induction of oxidative stress, and impaired protein synthesis. The generation of ROS is a substantial contributor to cadmium toxicity. cadmium depletes glutathione and antioxidant enzymes and blocks the electron transport chain in mitochondria, resulting in an increase in ROS generation in the body. cadmium toxicity is thought to be dose- and time-dependent, and ROS production is thought to be a mediator of the autophagic and apoptotic pathways (Zhang et al., 2021).

The apoptotic cell death produced by cadmium in JB6 cells was discovered to be reliant on Ca2+ - and H_2O_2-mediated JNK and p53 signaling, respectively (Son et al., 2010). Moreover, cadmium-induced autophagic cell death was also promoted through the ROS-dependent LKB1-AMPK pathway in response to the metal. Furthermore, cadmium causes necrotic cell death in skin epidermal cells (JB6), in addition to apoptosis. Cadmium poisoning causes ROS to be produced, which raises intracellular calcium (Ca21). Cadmium-induced apoptosis is hypothesized to be triggered by a surge in free calcium ions, which induces the creation of H_2O_2 in cells. Cadmium-produced H_2O_2 activates the activator protein-1 (AP-1) and p53 pathways in JB6 cells, but H_2O_2 from other sources does not (Son et al., 2011).

Cadmium induces apoptosis primarily via the Ca21-JNK and DNA-damaged inducible protein (GADD45) signaling cascades (Son et al., 2011). However, other pathways, including the H_2O_2-AP-1-p53-apoptosis-inducing factor (AIF) signaling

cascades, are also involved (J. Liu et al., 2009). Ca21 also causes the activation of MAPKs in mesangial cells exposed to cadmium and in human T-cell lines that have been treated with tributyltin. Researchers have discovered that cadmium at concentrations of 120 μmol/L and 10 and/or 20 M caused neurons to die *via* activating MAPK and mTOR, as well as the JNK and the PTEN-Akt-mTOR network, among other pathways (Chen, Liu, Luo, & Huang, 2008). In order to generate ROS, cadmium toxicity activates NADPH oxidase, which in turn activates MAPK activity. Ca21-dependent protein kinase and PI3K are also activated after cadmium toxicity, boosting MAPK. GADD45 is activated by phospho-p38-MAPK and caspase-3 is activated in the cell, resulting in apoptosis. Cytochrome c is released from the mitochondria and caspases are activated, resulting in apoptosis (Yiming et al., 2021).

mTOR is a vital element in the biosynthesis of proteins (X. Wang & Proud, 2006). L-Carnitine supplementation has previously been shown to increase protein deposits in fish; this may be due to L-Carnitine's ability to increase the lipid-sourced supply of energy, which reduces protein catabolism for energy usage. Dietary L-Carnitine, however, has been shown in certain studies to have a detrimental impact on protein deposition. The capacity of L-Carnitine to perform distinct functions in protein deposition across a variety of studies continues to be a critical unanswered subject (L.-Y. Li et al., 2020). However, none of the previous studies was indicated specifically for rat liver; that is why, in this experiment, the expression of L-Carnitine and cadmium was evaluated to discover how it affects rat liver and how it interacts with the mTOR pathway.

CHAPTER II

Materials and Methods

Ethical approval was granted by the Near East University Animal Experiments Local Ethics Committee Board (YDU/2022/01-147).

The information provided in this chapter highlights the materials and processes involved in the experiment. The experiments aimed to evaluate the expression of *mTOR*, *Akt1*, and *Akt2* in rat livers given cadmium and L-Carnitine and to provide a further understanding of the mechanism involved in how cadmium and L-Carnitine interact with the mTOR pathway.

2.1 Sample Collection and Sample Size

The rats were bred in the Near East University Experimental Animals Facility, and they were dissected, and the livers were stored in a -80° freezer. A total of 24 female rats was divided into four groups. The first group was administrated cadmium 2 mg/kg twice a week. The second group was administrated cadmium 2 mg/kg twice a week and L-Carnitine 75 mg/kg daily. The third group was administrated L-Carnitine 75 mg/kg every day. The fourth group was the control group. Thus, nothing was administrated to these rats. The rats were administrated these drugs orally for 28 days.

2.2 Analysis of the Liver Samples: RNA Extraction, cDNA Synthesis, and Real-Time PCR

The study included four rat groups, cadmium group, cadmium and L-Carnitine group, L-Carnitine group, and the control group, respectively. This experiment was conducted at NEU DESAM Research Institute laboratory, Nicosia, North Cyprus.

RNA Extraction was performed using the Hibrigen total nucleic acid isolation kit (Hibrigen, Turkey, cat. No. MG-TNA-01-10) following the manufacturer protocol. The first step in RNA extraction is washing the samples with PBS buffer. The samples are subsequently split into smaller pieces and centrifuged. The lysis buffer is then added and the samples are vortexed to blend with the lysis buffer. After that, the RNA is kept from degrading by incubating it in ice and then adding chloroform. After vortexing the samples and centrifuging them again, the DNA is separated from the chloroform using

ethanol. Finally, the solution is placed into columns and centrifuged, and the DNA is washed.

The purity of the extracted RNA was estimated using the Nano-drop Spectrophotometer (Thermo-scientific, Pittsburg, USA). For cDNA synthesis, three kits were used. The first one is the Norgen TruScript First Strand cDNA Synthesis kit for mRNA (Norgen, Canada, product no. 54400), the second is Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland, cat. No. 04 896 866 001), and the third one is Hibrigen cDNA synthesis kit (Hibrigen, Turkey). The general protocol of cDNA synthesis includes adding the reaction mix, enzyme, RNA, and nuclease-free water. All kits were used to reverse the transcription from RNA to synthesize cDNA following the protocol of the manufacturers with no modifications.

The LightCycler® 480 SYBR Green I Master kit (cat. No. 04 707 516 001) was utilized for the real-time PCR following the manufacturer protocol without any modifications. The final concentration of 0.2 µM of the primers was chosen for forward and reverse primers as determined by the optimization procedure explained below. The primers were designed prior to the experiment by Assoc. Prof. Pinar Tulay. Primer sequences are listed in Table 2.1.

Real-time PCR Rotor-Gene Q (Qiagen) was used to examine the expression of the desired genes in the cDNA samples. Real-time PCR was set up according to the conditions listed in Table 2.2. In order to make sure the reaction run efficiently with no contamination, a negative control consisting only of the SYBR master mix was also run in the real-time PCR.

2.3 Optimization of PCR

During real-time PCR optimization, Pten primer was used in a test run, in which the total reaction volume was 10 µl containing SYBR Green master mix, primer (forward primer and reverse primer), nuclease-free water, and cDNA sample. Two different primer concentrations; 0.1µM and 0.2 µM, were tested. The test has shown that 0.2 µM was the optimal primer concentration for the experiments. Therefore, n this primer concentration was used.

2.4 Statistical Analysis

The statistical analysis of the samples was done using GraphPad prism v8.4.2.

Table 2.1

List of Primers Sequences and Their Melting Temperature.

Genes	**Forward Primer**	**Reverse Primer**	**Tm C^0**
mTor	CGCTTCTATGACCAGCTGAA	TGACAACTGGATCGCTTGAG	56 C^0
Akt1	GGCAGGAAGAGACGATG	CCTGTGGCCTTCTCTTTCAC	55 C^0
Akt2	GAAGACTGAGAGGCCACGAC	CTTGTAATCCATGGCGTCCT	56.6 C^0

Table 2.2.

Real-Time PCR Conditions.

	PCR Steps	**Temperature C^0/Time**	**Cycles**
Steps	Initial Denaturation	95^0 C / 10 minutes	1
	Denaturation	95^0 C / 10 seconds	40
	Annealing	55-56.6^0 C / 30 seconds (Depending on the primer used)	
	Elongation	72^0 C / 30 seconds	

CHAPTER III

Results

This chapter presents the results of the conducted experiment, the results are obtained from real-time PCR and were further analyzed using the one-way ANOVA statistical analysis method to create the following numerical and graphical results that are provided in this chapter.

A total of 24 liver samples acquired from female rats was divided into four groups; cadmium, L-Carnitine, cadmium and L-Carnitine, and the control group, respectively. The table below (Table 3.1) shows the RNA purity in this study. The results presented show the RNA concentration level range is 7856.7ng/µl given that the highest concentration was 8059.1 for sample 20, and the lowest concentration was 202.4ng/µl for sample 14. The mean value for RNA concentration yielded a result of 2723.7 ng/µl. Nucleic acid purity may be assessed by measuring the ratio of their absorbance at 260 and 280 nm (A260/280), which is an attribute of their concentration. The 260/280 ratio of pure RNA is 2.0 when compared to other nucleic acids. In part due to the fact that proteins have an absorbance of 280 nm, these ratios are routinely used to evaluate the amount of protein contamination that remains after the nucleic acid separation procedure has been successfully completed. Table 3.1 further indicates that all the samples were pure except for samples 1, 2, and 18, which showed the 260/280 ratio to be less than 2.0.

The statistical results of the experiments carried out on livers were obtained from female rats. ANOVA (analysis of variance) analysis was used to further analyze and evaluate the gene expressions within the four groups, and the CT value derived from the RT-PCR (Table 3.2). ANOVA analysis is implemented to analyze the differences of the means between the models, to acquire detailed statistical information from the recorded statistical results of the experiments carried out on the female rat liver samples. In addition, student’s T-test analysis was used in order to determine if there is a significant difference between two samples. T-test determines their significant difference by analyzing the mean and standard deviation.

By using the analysis tools, it eased the process of conveying the experiment to be significant or non-significant. In this case, the results provided insignificant results, due to no difference in the genes investigated, even after comparing them with the control group. The following graphs created from the results obtained from the ANOVA analysis tool, will describe and indicate the insignificance of the results. However, the results showed no significant difference in the expression level of genes investigated when compared to the control group. The cycle threshold (CT) values derived from the RT-PCR for each liver sample investigated are shown in table 3.2. Furthermore, the outcome of CT values of samples 19 and 20 in *Akt1* control group were duplicated. In both instances, the CT value outcome was 0. Getting a CT value of 0 indicates that there could be an error.

Table 3 .1

Nano-Drop spectrophotometer results for the concentration and the purity of the extracted RNA.

Sample ID	**Sample name**	**RNA concentration (ng/µl)**	**260/280 ratio**
1	L-Carnitine liver	5147.7	1.92
2	Cadmium liver	4912.1	1.98
3	L-Carnitine liver 1	3030.1	2.13
4	L-Carnitine liver 2	3131.2	2.11
5	Cadmium liver 1	2078.7	2.06
6	Cadmium liver 2	2814.5	2.09
7	Cadmium liver 3	2669.3	2.13
8	Cadmium liver 4	4120	2.05
9	Cadmium liver 5	550.8	2.07
10	L-Carnitine + Cadmium liver 1	1174.3	2.1
11	L-Carnitine + Cadmium liver 2	1527.8	2.11
12	L-Carnitine + Cadmium liver 3	276.4	2.05
13	L-Carnitine + Cadmium liver 4	206.8	2.06
14	L-Carnitine + Cadmium liver 5	202.4	2.07
15	L-Carnitine + Cadmium liver 6	446.4	2.04
16	Control liver 1	4257.8	2.04
17	Control liver 2	3754.6	2.08
18	Control liver 3	5171.5	1.92
19	Control liver 4	2637.7	2.07
20	Control liver 5	8059.1	2.11
21	Control liver 6	507.9	2.07
22	L-Carnitine liver 4	3242.2	2.11
23	L-Carnitine liver 5	3518.2	2.09
24	L-Carnitine liver 6	1930.9	2.06

Table 3.2.

Mean Ct values of RT-PCR results for each gene investigated.

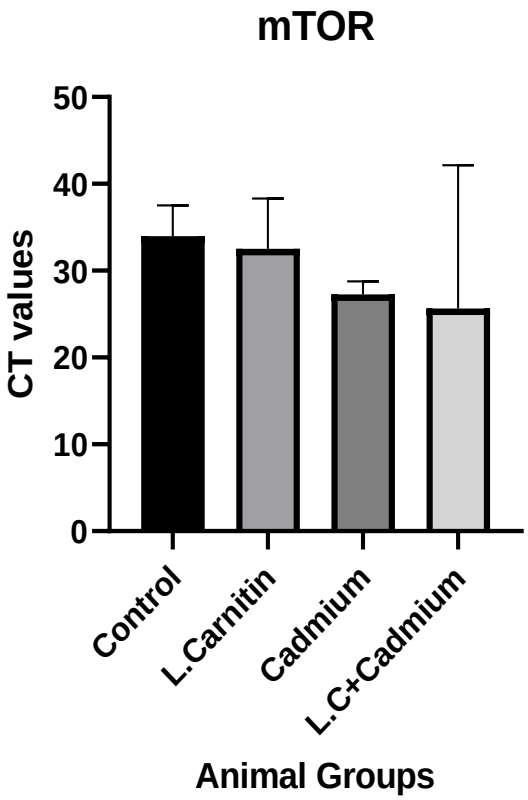
Sample ID	**mTOR**	**Akt1**	**Akt2**
1	27.71	31.75	31.32
2	29.58	28.73	35.85
3	27.3	31.47	32.15
4	26.98	28.35	36.5
5	26.17	29.55	33.99
6	28.15	29.62	32.54
7	25.85	30.23	30.68
8	25.9	27.06	32.29
9	27.9	30.4	32.53
10	33.98	34.65	13.17
11	36.38	36.25	37.55
12	9.57	35.88	35.5
13	38.47	34.5	36.99
14	35.39	32.14	14.57
15	0	37.33	35.87
16	29.91	18.42	33.81
17	29.63	36.78	33.16
18	35.55	39.37	36.12
19	36.81	0	38.48
20	37.97	0	37.16
21	34.07	21.27	35.31
22	38.92	19.55	0
23	36.21	37.68	35.51
24	38.09	0	38.42

3.1 mTOR Expression Analysis of Rat Liver

In this study, the levels of gene expression in liver samples obtained from female rats were evaluated using one-way ANOVA analysis. The findings of the investigation are depicted in Figure 3.1. In the case of the *mTOR* gene, the CT value of the control group was approximately 40, whereas L-Carnitine has a CT value slightly above 30, cadmium has a CT value slightly below 30, and L-Carnitine and cadmium group has a CT value slightly below 25, as demonstrated in the graph below. Since the p value was 0.3279, the statistical results were presented as non-significant ($p>0.05$). On the other hand, further comparisons with the control group using t-test revealed non-significant results.

Figure 3.1.

The statistical analysis of the CT values of mTOR in each one of the rat groups.

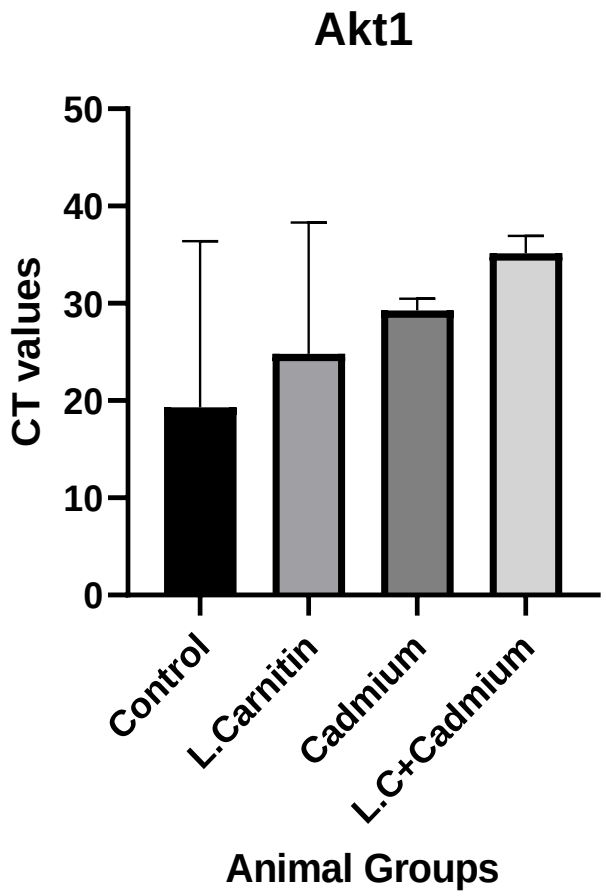


3.2 Akt1 Expression Analysis of Rat Liver

According to figure 3.2, one-way ANOVA was also used to determine the level of gene expression for *Akt1* gene. The CT value of the control group was approximately 20, whereas L-Carnitine was approximately 25, cadmium was approximately 30, and L-Carnitine and cadmium was slightly below 35 in the experimental groups. The p value was 0.113, the results were statistically non-significant. Furthermore, the differences between the groups were not statistically significant, and subsequent comparison with the control group using t-test produced non-significant results as well.

Figure 3.2.

The Statistical Analysis of the CT values of Akt1 in each one of the rat groups.

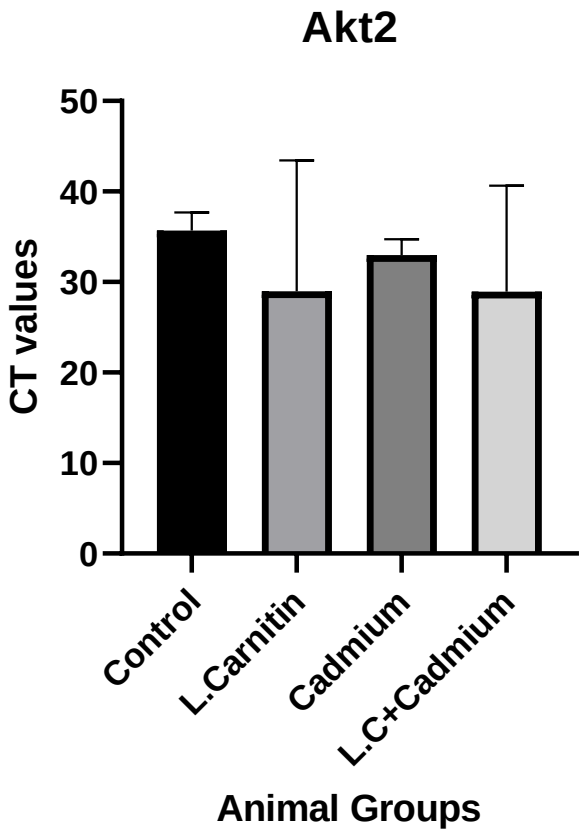


3.2 Akt2 Expression Analysis of Rat Liver

The statistical differences between the groups were recorded as p=0.5436, due to that, the differences between the groups were declared non-significant. As shown in figure 3.3, the CT value of the control group was approximately 35, the CT value of the L-Carnitine group was slightly below 30, the CT value of the cadmium group was slightly above 30, and the CT value of the L-Carnitine and cadmium group was approximately 30. Nonetheless, a more in-depth comparison with the control group using t-test yielded results that were not significant.

Figure 3.3.

The Statistical Analysis of the CT values of Akt2 in each one of the rat groups.



CHAPTER IV

Discussion

In this study, intake of different drugs administrated to rats was evaluated to explain the effect on the levels of gene expressions. The drugs used in the experiment are L-Carnitine and cadmium. To evaluate the effects of those drugs the experiment was indulged by creating four groups, a control group, given none of the drugs, L-Carnitine group, cadmium group, and L-Carnitine and cadmium group, respectively. This section is divided into the discussion of these drugs separately and how they can impact the levels of gene expressions by connecting their effects and functions relative to the mTOR pathway. Furthermore, within this section, the experiment is explained in detail, how the experiment is relevant, how it connects with other previous experiments, and the results found.

Primarily, cadmium is a hazardous toxic metallic element that can be harmful to all living creatures. There are many ways for cadmium to be introduced into the human body. Cigarette smoking and inhaling in addition to consuming contaminated food and water, are considered to be the most common reasons for cadmium to be attained. According to epidemiological evidence, exposure of cadmium in the environment may be linked to a wide range of malignancies, notably breast, prostate, pancreatic, and kidney cancers. It was further revealed that exposure to cadmium in the environment could increase the susceptibility to osteoporosis. The kidneys and liver are particularly vulnerable to the harmful effects of cadmium because of their high sensitivity to the toxin. Cadmium-induced proteins called metallothionein (MT) protect cells by attaching tightly to hazardous cadmium ions, and this protein synthesis capacity is considered to be reliant on the synthesis potentials of body cells (Biesecker, 2001).

Furthermore, there is indirect evidence that free radicals are involved in long-term cadmium toxicity and carcinogenesis in laboratory animals, after acute exposure to cadmium. Most scientists agree that the development of acute cadmium toxicity is accompanied by a significant increase in oxidative stress. Following an acute cadmium exposure, ROS are produced, which causes tissue injury. It has been shown that when people are exposed to long-term cadmium, their ROS production is reduced. Gained

cadmium tolerance, on the other hand, is associated with gene expression abnormalities that have a major effect on the formation of chronic cadmium toxicity and tumors (Patra et al., 2011).

Secondly, whether L-carnitine is ingested through the diet or generated in the kidneys and liver, must be highly concentrated in order for it to be used. It has been discovered that L-carnitine has a strong affinity for muscle and cardiac cells, as well as for cultured fibroblasts. There is a low affinity for L-carnitine receptors in the liver and brain, whereas there is an intermediate affinity for L-carnitine receptors in the intestinal epithelium and renal tubular cells (Arenas et al., 1998).

Among the enzymes that require L-Carnitine as a cofactor are the carnitine translocase and the acylcarnitine transferases I and II. Free long-chain fatty acids must be converted to acylcarnitines using these enzymes before they can be transported into the mitochondrial matrix. When the body lacks L-Carnitine, free fatty acids build up in the cytoplasm, causing damage to the cells and preventing the mitochondria from using the fatty acids for energy, resulting in an energy deficit (Goa & Brogden, 1987). L-Carnitine is essential for the last stages of glucose metabolism and may even modulate the mitochondrial respiration chain, according to a number of recent studies. In the oxidation of pyruvate, L-Carnitine may boost the activity of the pyruvate dehydrogenase complex (PHD). Additionally, studies have shown that supplementing with L-carnitine increases the activity of respiratory chain enzymes in muscle, and this is assumed to be due to mechanisms involving mitochondrial DNA (Arenas et al., 1998).

Notwithstanding the controversy surrounding the supplement, L-Carnitine has been presented as an effective therapy for a variety of disorders, including liver damage. According to the findings of several research, L-Carnitine supplementation can help to attenuate or protect against liver damage induced by a range of different conditions, including excessive alcohol intake. An increasing number of animal studies have indicated that dietary supplementation with L-Carnitine can help to prevent hepatitis and the development of hepatocellular carcinoma in rats, as well as help to reduce the liver damage induced by excessive alcohol intake in these animals. L-Carnitine has also been found to protect the membranes of liver cell membranes from oxidative changes in rats

that have been exposed to ethanol in their diet. This is owing to the fact that it has the ability to act as a free radical scavenger. Therefore, due to the antioxidant qualities of L-Carnitine, it has the potential to be utilized in the treatment of liver illnesses (Li et al., 2012).

Given the abundance of information on cadmium toxicity, which has been associated with increased ROS production and apoptosis, in addition to L-Carnitine as an antioxidant and a treatment for liver injury, it is important to address the interaction of these parameters with the mTOR pathway. mTOR integrates signals from upstream such as energy levels and stress in addition to growth factors and nutrients. As a result, one of the most important functions of mTOR is to integrate these signals into actions about whether or not to impact cellular proliferation, survival, and growth in a positive or negative manner. Energy level, as well as growth factor stimulation, have an effect on mTOR activity through the TSC2-Rheb signaling pathway. PI3K-AKT is responsible for most of the regulation of mTOR signaling by growth factors, such as insulin and IGF-1. AKT is activated when the insulin receptor is activated, which increases the levels of PIP3 in the blood and causes the activation of the enzyme PI3K. It has been shown that overexpression of active PI3K or AKT results in increased phosphorylation of the translational regulators S6K1 along with the eukaryotic initiation factor 4E binding protein 1 (4EBP-1), both of which are major mTOR targets in the regulation of translation. Furthermore, the phosphorylation of TSC2 by PI3K-AKT is the primary mechanism by which mTOR is regulated. It has been shown that TSC2's capability to inhibit mTOR is elevated as a result of the loss of AKT phosphorylation sites. This, in turn, results in higher S6K phosphorylation. Activation of mTOR requires the phosphorylation of TSC2 by AKT, and the inhibition of TSC2 GAP activity against Rheb is required for mTOR pathway activation. Nevertheless, it is still unclear whether AKT-mediated phosphorylation of TSC2 GAP activity is a direct effect on GAP activity (Lee et al., 2007). To gain a better understanding of the medications involved on an individual level, as well as the system of mTOR pathways impacted by those drugs, previously published studies that were comparable in nature have been investigated intensively.

In a study that was conducted to investigate the effect of cadmium on the mTOR pathway in neuronal cells, researchers discovered that the outcomes of activating the Akt/mTOR pathway under various stress conditions are entirely different. Evidence suggests that the survival of neuronal cells requires a particular level of mTOR activation. Moreover, cadmium caused apoptosis in neuronal cells by generating ROS which consequently led to activating MAPK and mTOR signaling pathways in PC12 as well as SH-SY5Y cells according to their findings (Ueno et al., 2015).

In another study conducted by Zhu and colleagues (2021), cadmium was found to have an effect on the cell cycle, proliferation, and apoptosis in granulosa cells from laying hens by regulating the MAPK, AKT/FoxO3a, and mTOR pathways (Zhu et al., 2021). A further finding was that cadmium altered microRNA gene expression levels, which may regulate signaling pathways such as AKT/ERK1/2/mTOR and cell cycle progression by regulating the expression of G proteins as well as the expression of proteins that related to the cell cycle. Another previously published study has demonstrated that upon exposure to cadmium in primary rat cerebral cortical neurons and PC12 cells, it resulted in ROS-dependent activation of the Akt/mTOR signaling pathway, which ultimately resulted in apoptosis of the neurons (Yuan et al., 2016).

A dietary L-Carnitine supplementation research in zebrafish, on the other hand, found that it may increase liver and muscle carnitine concentrations and reduce both organs' lipid contents by improving mitochondrial oxidation and decreasing fat synthesis. Consumption of dietary L-Carnitine may potentially have an effect on protein synthesis through increasing the *mTOR* gene expression in the liver, which is consistent with previous findings (J.-M. Li et al., 2017). However, according to Jang and colleagues (2016), the Akt/mTOR pathway and L-carnitine did not have any interaction (Jang et al, 2016). L-Carnitine, on the other hand, has been shown to increase Akt1 and mTOR protein phosphorylation in the quadriceps femoris muscle of developing rats (Keller et al, 2013).

The supplementation of L-Carnitine has been shown to significantly raise the levels of plasma of IGF-1 in studies conducted on both humans and animals (Keller et al., 2013). The Akt/mTOR signaling pathway, which is activated by IGF-1, is responsible for

protein synthesis. The downstream signaling pathway is activated as a result of the binding of IGF-1 to the receptor on its surface (J.-M. Li et al., 2017).

In contrast to the previously conducted studies, this study, involving liver samples acquired from female rats, that were administrated with different drugs, was used to evaluate the expression of three genes that are involved in the mTOR pathway (*mTOR, Akt1, and Akt2*). The results of the statistical analysis using one-way ANOVA were considered to be non-significant when compared to the control group individually using t-test. Furthermore, when comparing the cadmium group with L-carnitine and cadmium group, an unpaired result was shown and a p value of 0.0001 was given in the *Akt1* gene group. This is indicating a statistically significant difference between cadmium group and cadmium and L-carnitine group due to the fact that the p value was <0.05. L-carnitine has been shown in earlier studies to increase the levels of IGF-1, which may account for the statistically significant difference. High levels of IGF-1 cause the activation of *Akt1*, which then causes the activation of the mTOR pathway.

The statistical analysis displayed a high standard deviation. The standard deviation is a measure of how widely dispersed the data is in relation to the mean. When the standard deviation is low, it indicates that the data are close to the mean. The higher the standard deviation the more dispersed and unreliable the data are. As presented in figure 3.1, L-Carnitine and cadmium group has a high standard deviation, which may be due to the weight differences between the rats, or due to some technical errors such as the partial loss of drugs due to unfactored and sudden movement of the rats during the intake of the drugs. Furthermore, as shown in figure 3.3, the standard deviation is also high in L-Carnitine group as well as L-Carnitine and cadmium groups due to the same previous reason concerning the loss of some drug portions during intake, as well as the physical differences between the rats (such as the bodyweight). Moreover, the results of the CT values of samples 19 and 20 in Akt1 control group (figure 3.2) were replicated to determine the outcome. In both cases, the CT value resulted in a value of 0. Getting a CT value of 0 implies that there is a possibility of a problem. Real-time PCR was performed once again to ensure that the error had been identified. In the second trial, the SYBR green mix preparation procedure was repeated. The fact that the identical data were

withheld from the experiment shows that the technical mistake occurred during the RNA extraction or cDNA synthesis. Due to the lack of time and the absence of liver samples, the RNA extraction and cDNA synthesis procedures were not repeated.

CHAPTER V

Conclusion and Limitations

This study evaluated the expression of genes associated with the mTOR signaling pathway. The liver samples used in this study were collected from 24 female rats who were divided into four groups and administrated with specific drugs prior to the experiment. The study concentrated on two drugs for the reason that those two drugs have been proven in previous research studies to have effects on both animal and human tissues. Previous studies concluded that cadmium-induced toxicity increases ROS production, which leads to inducing apoptosis through the mTOR pathway. In contrast, previous studies also proved that L-Carnitine has the ability to work as a free radical scavenger as well as being part of liver injuries treatment. Furthermore, L-Carnitine enhances protein synthesis, through indirectly activating the mTOR pathway by activating IGF-1. Even though, the information provided about the drugs is from previous experiments is believed to be certain. As the study on the liver samples of the rat was conducted, it was taken a bit further to test those drugs separately in groups and also have one group of rats take both drugs as a combination. As known, the drugs will have different effects on the liver, as one is toxic and the other is beneficial. However, the negative outcome of cadmium is expected to be crossed out with the positive outcome of L-carnitine, but considering the gene-gene interactions and the correlation between pharmaceutical effects and genes can be unpredictable. Therefore, the experiment expected a significant outcome regarding gene expression evaluation, mostly in the rats that were given both drugs.

A number of limitations are noted in this study. These limitations are the sample size, along with the inaccuracy of the drug concentrations due to differences in rats' body weight and the duration of the experiment. These limitations could make the experiment more accurate if taken into consideration. Firstly, larger sample size will yield more accurate and reliable results, due to the fact that testing more samples will lead to a more decisive evaluation, however, using a larger number of rats is unaccepted due to ethical conflicts. Secondly, based on standard deviation there could be some differences between the rats such as body weight in addition to some technical errors such as lost portions of

the drugs during intake, to overcome this limitation, a precise evaluation of the rats must be done to determine the ideal concentration of the drugs. The intake of the drugs can be enhanced to prevent any lost portions of the drugs. Lastly, the extension of the duration from 28 days, will create a more accurate result, allowing the rat body to absolve the drugs. The limitations played a big part in the outcome of the experiment as explained, due to that, the concluding results of the experiment were insignificant.

In conclusion, no studies have been conducted on rat liver samples to determine the involvement of cadmium and L-Carnitine in the mTOR pathway-related genes (*mTOR, Akt1, and Akt2*). According to the limitations involved in the study and the results evaluated, there are no significant differences between the rat groups that were administrated with the drugs even when compared with the control group using t-test.

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