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The Effect of Rapamycin on the Cyclin D Expression in Some Tissues of Swiss Albino Mice Mus Masculus Embryos

Sada Ghalib Taher[1], Muntadher Lutfi Taher[2], Riyadh Rashid Hameed[3], Ali Naeem Salman[4], Nahla A. Al-Bakri[5]

[1]College of Dentistry, University of Thi-Qar, Thi- Qar-64001, IRAQ

[2]College of Health and Medical Technologies, National University for Science and Technology, Thi- Qar-64001, IRAQ.

[3]College of Health and Medical Technologies, National University for Science and Technology, Thi- Qar-64001, IRAQ.

[4]University of Thi-Qar College of Education for pure sciences Department. of Biology.

[5]University of Baghdad, College of Education for Pure Science (Ibn Al-Haitham), Department of Biology.

ABSTRACT

The current study aimed to investigate the effects of Rapamycin (Rapa) on the cyclin D expression in heart and liver tissues of Swiss albino mice Mus masculus embryos at different Embryonic Developmental stages. The study conducted on Thirty- two pregnant albino mice, were randomly divided into four groups, each group contained eight pregnant mice. Each group received Rapamycin (Rapa) at different doses, via intraperitoneally injection (IP), at different gestational days until the conclusion of the designated times, whereas the control groups received a DMSO. Pregnant mice were administered the desired doses under the same environmental circumstances. According to pharmacological constitutions, body weight was used to establish the dosages. After that, on days 13^{th} and 16^{th}, the mice were put to killing. Current study used another two concentrations from drug, one less than the therapeutic dosage (0.75mg/kg of Rapamycin) and another more than the therapeutic dosage (3mg /kg of Rapamycin). Quantification of the IHC of cytoplasmic proportion and intensity showed cytoplasmic expression of Cyclin D in tissue of mice embryos heart the control group and other treatment groups differed significantly from one another at ($P < 0.05$), while there wasn't a significant difference between the treatment groups which received 0.75 and 1.5mg/kg of Rapamycin, in embryonic liver tissue. At gestation day 16^{th}, there was no significant difference in expression of Cyclin D between control group and treated group with 0.75mg/kg of Rapamycin, while there was a significant difference in Cyclin D expression at ($P < 0.05$) between treated groups with 0.75mg/kg of and treated group with 1.5 and 3 mg/kg of Rapamycin and from these results we concluded that the use of Rapamycin has been affected on checkpoints of cell cycle during embryogenesis, which appeared through its effects on cyclin D expression.

KEYWORDS: Rapamycin, Cyclin E, Cyclin D, heart, liver, Albino Mice Embryos.

ARTICLE DETAILS

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I. INTRODUCTION

Rapamycin (Rapa) is a macrolide antibiotic and immune modulator that was first discovered in the 1960s and was finally authorized for the prevention of solid organ transplant rejection by the Food and Drug Administration (FDA) in 1999.It is efficient in preventing allograft rejection and has anticancer effects (law,2005). The antifungal agent Rapamycin was initially identified to be produced by the bacteria Streptomyces hygroscopicus. This substance was discovered to have antiproliferative and immunosuppressive effects on mammalian cells shortly afterward, iImmunosuppressive drugs are used to treat a number of different of inflammatory and autoimmune diseases (Leroy et al., 2015).

Cyclin D1 is a fundamental regulator of the cell cycle (Vartanian et al., 2011). It is a key regulator of the G1 phase of the cell cycle, promoting cells in transitioning from G1 to S (Stacey,2003).When cyclin D1 function is disturbed, cell cycle arrest occurs in the G0/G1 cell cycle phase (Liu et al., 2020).

"The Mammalian Target of Rapamycin (mTOR)", is a protein kinase possesses a molecular weight of 290 kDa and is an atypical serine threonine protein kinase, mTOR regulates protein synthesis, mitochondrial function, autophagy, cytoskeleton organization, and cell survival processes (Morad,2010).To coordinate cellular development and metabolism, mTOR integrates environmental signals such as amino acids, growth factors, and cellular energy such as ATP (Rion,2017).

During embryogenesis in vertebrates, the heart is the first organ to form and functioning (Olson,2006). Developmental stages of mouse heart can be divided into five overlapping stages: Heart tube formatoion stage (E8.5), the proepicardial organ (PEO) stage (E9.5), cushion formation, atrial and ventrical septation stage (E10.5-12.5), At (E13.5) out flow tract septation (Vegh et al., 2016), the initial morphological sign of the mice's liver development is the formation of the hepatic diverticulum, the liver bud has a period of rapid development between E10 and E15 (Taher,2022)

II. MARTIALS AND METHODS

A. Ethics statement:

The University of Southern China's Institutional Animal Care & Use Committee No. 2014 authorized the rules that were followed for all animal handling procedures (Wang et al., 2020).

B. Preparation experimental animals

The current research utilized female-albino mice from the Balb/c strain (Mus musculus) aged 11-12 weeks, ensuring that the subjects were in the prime of their health. These mice, obtained from the Animal House, were placed in the Biology Department at" Thi-Qar University's College of Education for Pure Science", where they were housed in the cages with sawdust-brushed metal lids. The mice were kept in an environment that was well-maintained and regulated, with consistent ventilation and temperatures between 20 and 24 C°, and a 12-hour day \ night photoperiod cycle. Mice were taken to ensure their health and free from diseases the vet ,these measures were taken to ensure that the mice were in optimal conditions for the study, The animals received enough food and water from a locally source (Wheat 34% , corn 25% ,barley 20% , powdered milk 10%,animal protein 10% , 1% salt all material grinding and then mixing with some water and oil, until they become a paste coherent) then placed in the cages' designated food region. Two adult females were confined together with one mature male overnight, and the females were checked for the presence of a vaginal plug on the next morning(Purves and Lichtman 1985), On the cages, the date of mating was written which is Day zero of gestation (D0), the next day is the first day of gestation (D1st) (Damayanti etal., 2021)

C. Rapamycin preparation

Different doses Rapamycin was injected to the experimental animals. The mice were injected via Intraperitoneal injection, the different concentrations of Rapamycin were chosen according to the therapeutic dose (1.5 mg to 1 kg) (Wang et al., 2020). 32pregnant mice were divided into four groups according to an arrangement as follow:

1- The control group: consisting of 8 pregnant mice that were treated with DSMO by the intraperitoneally injection.

2- Experimental groups: involving 24 pregnant albino mice, divided into two group first group including 12 pregnant mice at the day 13th of gestation and the second group was dived into three sub group, including 12 pregnant mice at the day 16th gestation, treated with different doses of Rapamycin drug as follows:

i. The first, which treated with 0.75 mg \ 1kg from the body weight of Rapamycin.
ii. The second, which treated with 1.5 mg \ 1kg of Rapamycin.
iii. The third, which treated with 3 mg \ 1kg of Rapamycin.

The pregnant mice injection has started at the day 8th of pregnancy, embryos have been obtained and eliminated separately at age of the day 13th and 16th of gestation according to Wang etal., (2020).

D. Immunohistochemistry (IHC) assay:

Immunohistochemistry was used to stain the mice embryonic tissue sections using CCND1antibodies (Mouse monoclonal, dilution 1:250, MyBioSource, cat. number MBS175034.The preparation of IHC solutions and dyes, according to (Alghezi, 2019).

Before IHC, pretreatment procedures were applied. Embryonic tissue slices with embedded paraffin (5 µm) were cut, deparaffinized using Histoclear, and rehydrated using varying percentages of alcohol (100%, 95%, and 70%, respectively). After permeabilizing the tissue sections with 0.5% triton X-100 in PBS (phosphate buffer saline), they were heated to 90°C for 30 minutes to induce epitope retrieval in a citrate buffer (pH 6.0) containing 0.05% Tween 20. The tissue sections were then allowed to cool for 20 minutes. To inhibit endogenous peroxidase activity, drops of 3% H2O2(PathnSitu-peroxidase) were applied to the tissue segment in a humid room. Furthermore, drops of a solution made from the 10% normal goat serum and 0.05 bovine serum albumin (BSA), were applied to tissue slices in PBS.

The tissue sections (heart and liver tissues), were ready to stain with a PathnSitu IHC staining kit after completing the pre-treatment methods mentioned above. Primary antibodies were diluted to the proper dilution with PathnSitu antibody diluent, and 100-150 µ of diluted primary antibodies were applied to each section and incubated at 4C° overnight in a humid chamber, before being rinsed three times for ten minutes each. The tissue section was then covered with the secondary antibody the following day, and it was incubated for 30 minutes at room temperature. In accordance with the manufacturer's instructions, the EnVision+Kit (MyBioSource,UAS) was used as a chromogen to see the reaction products. The sections were then counterstained with hematoxylin stain (H-3401, Vector Laboratories,

Peterborough, UK). DPX was used to mount these sections (Sigma-Aldrich, Gillingham, UK). A Nikon Digital-Sight DS-U1 CCD digital camera was utilized for taking images, while a Nikon Eclipse E800 bright-field illumination was employed to see stained embryonic tissues.

E. Immunohistochemistry (IHC) Quantification

For each potential biomarker, Five fields were randomly taken at 40x and 100x magnification prior to measurement, the images were then evaluated using one of a variety of scoring techniques to determine the expression of the cyclin D in the samples tissues ,based on past research, the most appropriate IHC scoring system for each biomarker was determined.

F. Cyclin D Scoring

The cytoplasmic staining was scored by using a semi-quantitative scoring system as the following arrangement: the percentage of positive cells was scored as:"(0: 0; 1: 1-25%; 2: 26-50%; 3:51-75%; and 4: 76-100%), while the intensity was graded as (0: negative, 1: weak, 2: moderate; and 3: strong). The final scoring represents the summation of the proportion and intensity scores, which ranged from 0 to 7"(Alghezi etal.,2021).

G. The Statistical Analysis

The results were analyzed statistically using the program Statistical Package for Social Sciences (SPSS) and Excel ,by using the Least Significant Difference Test (LSD) among different groups in study and the differences were considered significant between the averages at the P-value ($P<0.05$) ,ANOVA and t-test .

III.RESULTS

Results showed many changes has been taken place in the heart and liver tissues of the embryonic mice which were treated with different concentrations of Rapamycin, these changes are shown as followings:

A. The effects of Rapamycin on Cyclin D Expression on the mice embryos' hart

1. Embryo at 13th day of Gestation.

Immunohistochemical staining showed negative nuclear and cytoplasmic staining at control group tissues (Figure1A), as well as at group treated with 0.75mg /kg (Rapa) (Figure 1B).A cytoplasmic staining was detected, when they treated with 1.5mg /kg (Figure 1C), the staining was stronger when they treated with 3mg/kg than others groups (Figure 1D).

2. Embryo at 16th day of Gestation (GD16)

The embryos heart tissue at 16th day of gestation revealed negative nuclear and cytoplasmic staining in control group(Figure 2A).Treated with 0.75mg/kg Rapa ,heart tissue showed moderate to strong cytoplasmic staining was identified (Figure 2B), at 1.5mg/kg showed moderate cytoplasmic staining, (Figure2C), when they were treated with 3 mg/kg Rapa, the staining was stronger in the placenta tissues because of the impossibility of obtaining embryos in this group due to growth restriction in the uterus resulting from treatment with Rapamycin (Figure 2D).

The statistical analysis examined first at the expression of Cyclin D in embryos heart tissues in control group and treatment groups (0.75, 1.5 and 3 mg/kg Rapamycin). Quantification of the IHC of cytoplasmic proportion and intensity of Cyclin D staining showed cytoplasmic expression of Cyclin D, at 13th day of gestation the results showed a significant difference between control group and other treatment groups at ($P \leq 0.05$), and there was no significant difference for Cyclin D expression between 0.75 and 3mg/kg of Rapamycin, there was a significant difference between control group and other treatment groups at ($P \leq 0.05$) at the day 16th of gestation, while there was no significant difference between the treatment groups 0.75 and 1.5mg/kg of Rapamycin (Table 1) .

Table1: Quantification of cytoplasmic proportion and intensity of Cyclin D staining in tissue of mice embryos heart.

Gestation day	Control	Rapamycin Concentration mg/kg		
		0.75	1.5	3
13th	0.00 ± 0.00^{a}	1.40 ± 0.28^{b}	5.35 ± 0.61^{c}	1.95 ± 0.41^{b}
16th	0.00 ± 0.00^{a}	5.30 ± 0.20^{c}	5.40 ± 0.28^{c}	6.15 ± 0.19^{d}
Two Way ANOVA P. value (Treatment & Period)	≤ 0.001**			
L.S.D	0.707			

Similar symbols in ANOVA indicate no significant differences.

*Significant according to $P \leq 0.05$

** Highly Significant according to $P \leq 0.01$

B. The effects of Rapamycin on Cyclin D Expression on the mice embryos' liver

1. Embryo at 13th day of Gestation

In the control group, immunohistochemical staining for Cyclin D in the embryo's liver tissue on day 16th of gestation revealed negative nuclear and cytoplasmic staining (Figure 3A) Liver tissue of embryos treated with 0.75 mg/kg Rapa, showed moderate cytoplasmic Cyclin D staining (Figure3B), while at 1.5 mg/kg Rapa group showed strong cytoplasmic staining (Figure3C). When they were given 3 mg/kg Rapa, the staining was stronger than another group (Figure3D).

2. Embryo at 16th day of Gestation (G16)

At the 16th day of gestation, immunohistochemistry staining for Cyclin D in the embryo's liver tissue revealed negative nuclear and cytoplasmic staining in the control group (Figure 4A) and at the group treated with 0.75 mg/kg Rapa (Figure 4B), but those treated with 1.5 mg/kg had significant moderate cytoplasmic staining (Figure 4C). The

staining was stronger in the 3 mg/kg Rapamycin group than in the other groups (Figure 2D).

The expression of Cyclin D in embryonic liver tissue was evaluated statistically. The cytoplasmic expression of Cyclin D was quantified using IHC of cytoplasmic proportion and intensity of Cyclin D staining at the 13th day of gestation ,there was no significant difference between in both control and 0.75mg/kg Rapamycin treatment groups, there was a significant difference in expression of Cyclin D between control group and the treated groups with 1.5 and 3 mg/kg at ($P \leq 0.05$) .At gestation day 16th there was no significant difference in expression of Cyclin D between control group and treated group with 0.75mg/kg of Rapamycin, while there was a significant difference in Cyclin D expression at ($P \leq 0.05$) between treated groups with 0.75mg/kg of and treated group with 1.5 and 3 mg/kg of Rapamycin (Table 2).

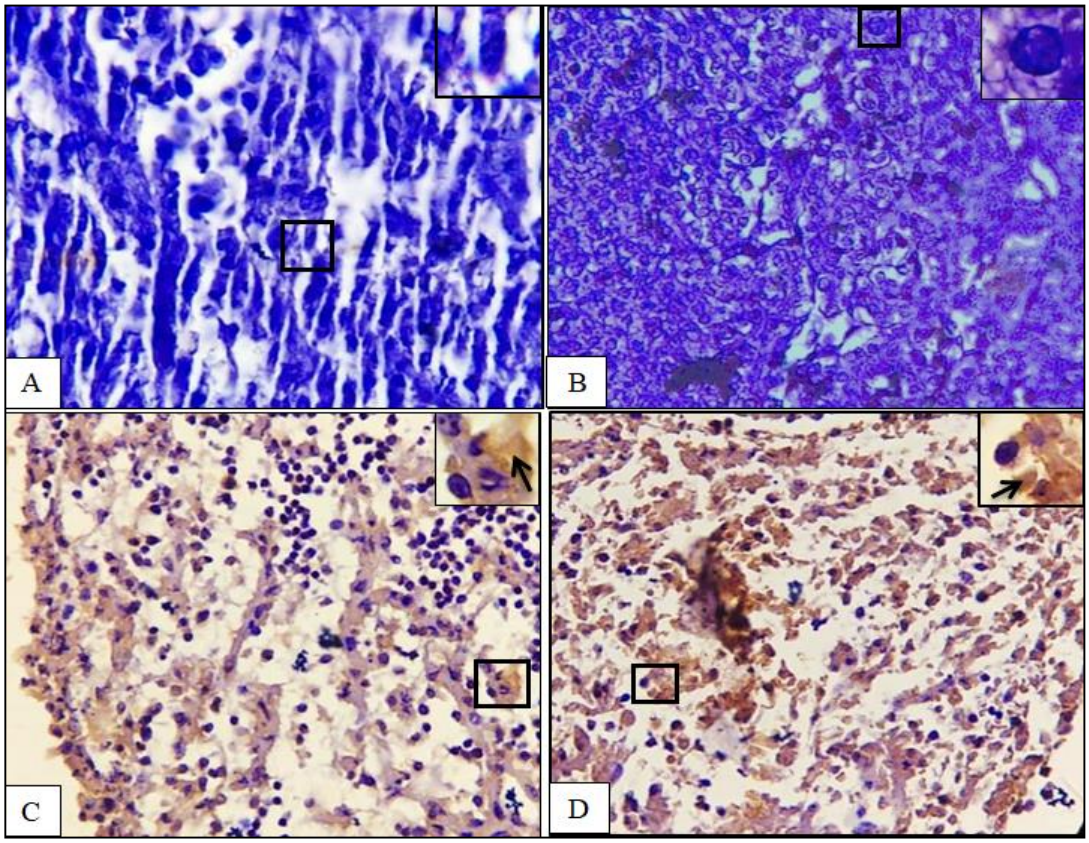

Figure1: Cyclin D staining in samples from longitudinal section of mice embryo's heart at day 13th of gestation day .**A**: control group shows negative nuclear and cytoplasmic Cyclin D staining, **B**: treated with 0.75 mg/kg Rapamycin, shows negative nuclear and cytoplasmic Cyclin D staining,**C**: treated with 1.5 mg/kg Rapamycin, Strong cytoplasmic Cyclin D staining**, D**: treated with 3 mg/kg Rapamycin, Strong cytoplasmic Cyclin D staining .**Black arrows** indicates cytoplasmic Cyclin D expression stained by DAPI .Magnification 100 x.

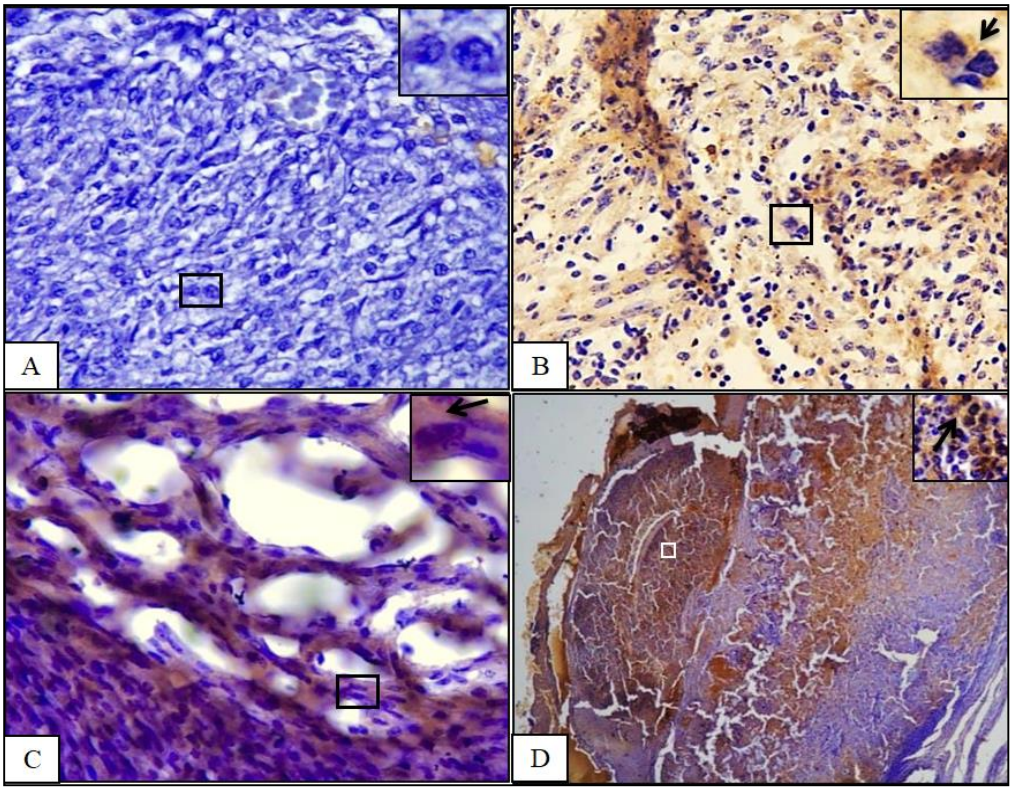

Figure 2: Cyclin D staining in samples from longitudinal section of mice embryo's heart at day 16th of gestation .**A**: control group shows negative nuclear and cytoplasmic Cyclin D staining, **B**: treated with 0.75 mg/kg Rapamycin, shows moderate to strong Cyclin D staining .**C:** treated with 1.5 mg/kg Rapamycin, shows strong cytoplasmic Cyclin D staining**, D**: treated with 3 mg/kg Rapamycin, degenerative embryo shows strong cytoplasmic Cyclin D staining .**Black arrows** indicates cytoplasmic Cyclin D expression stained by DAPI. Magnification 100 x.(**D:**10x . Magnification 100 x)

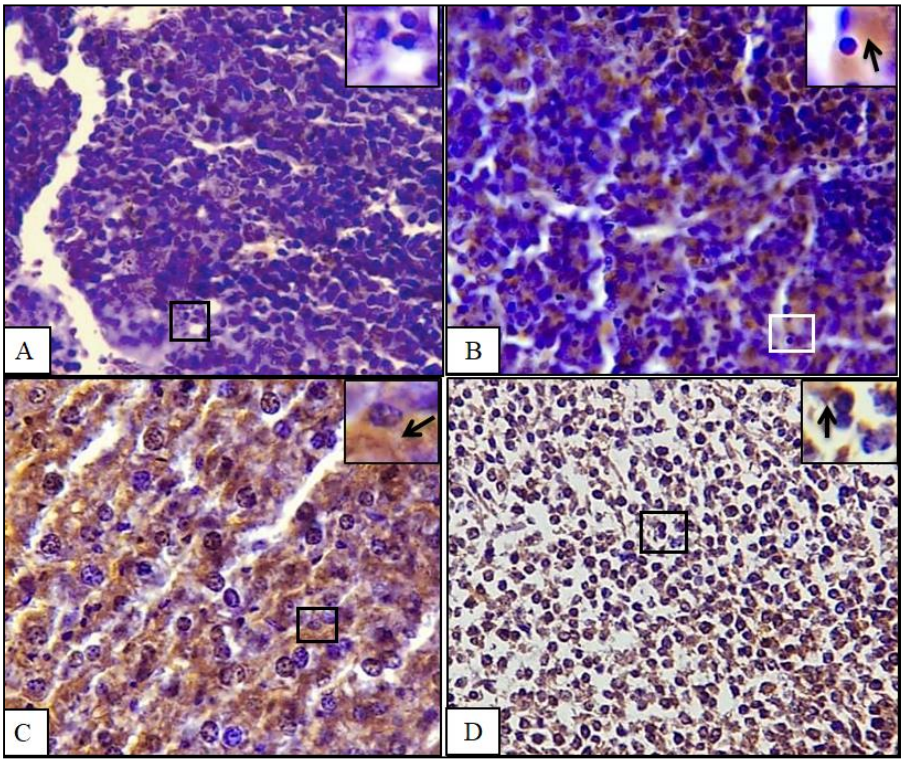

Figure 3: Cyclin D staining in samples from longitudinal section of mice embryo's liver at the day 16th of gestation .**A**: control group shows negative nuclear and cytoplasmic Cyclin D staining, **B**: treated with 0.75 mg/kg Rapamycin, shows moderate Cyclin D staining .**C:** treated with 1.5 mg/kg Rapamycin, shows strong cytoplasmic Cyclin D staining**, D**: treated with 3 mg/kg Rapamycin, shows strong cytoplasmic Cyclin D staining. **Black arrows** indicate cytoplasmic Cyclin D expression stained by DAPI. Magnification 100x.

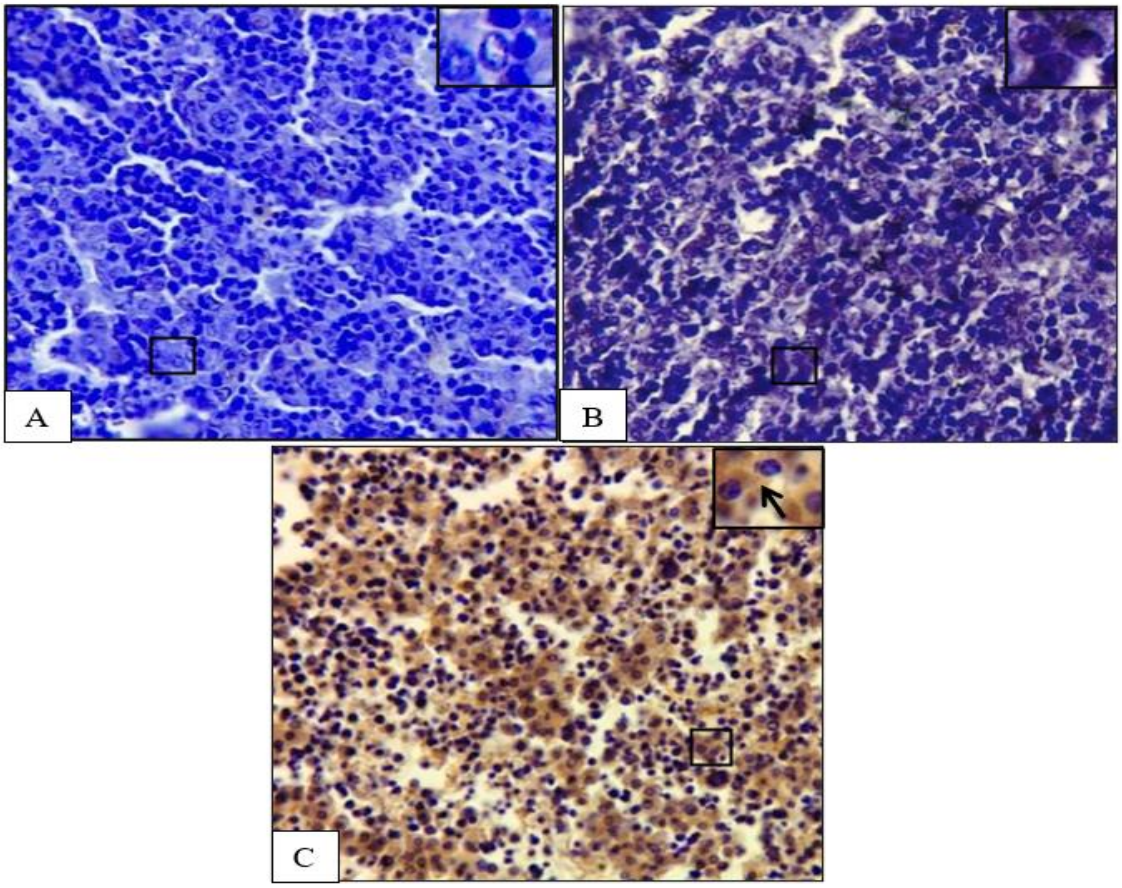

Figure 4: Cyclin D staining in samples from longitudinal section of mice embryo's liver at day 13th of gestation .**A**: control group shows negative nuclear and cytoplasmic Cyclin D staining, **B**: treated with 0.75 mg/kg Rapamycin, shows negative nuclear and cytoplasmic Cyclin D staining.**C**:treated with 1.5 mg/kg Rapamycin, strong cytoplasmic Cyclin D staining **Black arrows** indicates cytoplasmic Cyclin D expression stained by DAPI. Magnification 100x

Table 2: Quantification of cytoplasmic proportion and intensity of Cyclin D staining in tissue of mice embryo's liver.

Gestation day	Control	Rapamycin Concentration mg/kg		
		0.75	1.5	3
13th	0.00±0.00a	0.00±0.00a	5.85±0.30c	4.55±0.86b
16th	0.00±0.00a	0.00±0.00a	6.40±0.28c	6.15±0.19c
Two Way ANOVA P. value (Treatment & Period)	≤ 0.001**			
L.S.D	0.779			

Similar symbols in ANOVA indicate no significant differences**.**

*Significant according to P ≤ 0.05

** Highly Significant according to P ≤ 0.01

IV. DISCUSSION

Rapamycin causes a G1 cell cycle arrest in various cell types, which is associated with a decrease in Cyclin D1 levels (Hashemolhosseini et al., 2006) and Cyclin D1 overexpression restores the Rapamycin-induced cell cycle arrest that what was confirmed by Nelsen et al., (2003).

A sufficient supply of nutrients is necessary for cell cycle progression providing sufficient energy and the production of proteins, which facilitate cell growth, as the fundamental coordinator of nutrition availability and metabolism, mTORC1 controls energy homeostasis for the advancement of the cell cycle. (Cuyas et al., 2014). mTORC1 can alter Cyclin dependent kinases (CDKs), their obligatory Cyclin binding partners, and inhibitors of CDK, which govern cell cycle progression, in addition to controlling cellular metabolism.

Inhibiting mTOR activity with Rapamycin promotes G1-phase cell cycle arrest (Foster et al., 2010). G1/S transition Cyclins (D-type and E-type Cyclins) are regulated both transcriptionally and translationally, both downstream arms of the 4E-BP1 and p70S6K of the TORC1 signaling cascade are necessary to facilitate the progression of mTOR-dependent from G1 phase (Oka et al., 2013). Furthermore, AKT or SGK1 phosphorylates the CDK inhibitor p27kip1, a sequesters the protein in the cytoplasm by Phosphorylation of

p27kip1 blocking its nuclear role as a CDK inhibitor, allowing the Cyclin DeCDK4/6 complex to stabilize for the "G1/S transition" to occur (Medema et al., 2000).

In current study Rapamycin administration in higher concentrations cause overexpression of Cyclin D1 this may be return to restores Rapamycin-induced cell cycle arrest that was confirmed by Law et al (2006), the hypothesis which stand on "The regulation of hepatocyte development through the G1 phase is significantly influenced by the activation of the Cyclin D1/cdk4 kinase", Cyclin D has been clearly identified as a critical modulator of the progression of the hepatocyte cell cycle (Rickheim et al., 2002) , Therefore, earlier research indicates that Cyclin D1 expression is crucial for controlling hepatocyte proliferation, and this protein's expression seems to be enough to encourage the growth of these cells.

Current study defines the primary objective may be inclined to hypothesise that the control of Cyclin D1 expression may also account for other traits in mice, given the separate output from mTOR that governs Cyclin D1 promoter activity. In mammals, cyclin D plays a role in regulating cell size. (Nader et al., 2005). In addition, Cyclin D may also regulate the process of mitochondrial biogenesis (Wang et al., 2006).

Based on the following observations, it is likely that the down-regulation of Cyclin D1 expression largely contributes to the defective regeneration of S6K1-null livers, although other processes may also be implicated. First, Since cyclin D mRNA and protein levels are already decreased during rest, it appears that S6K1 activity targets Ccnd1(cyclin D) DIRECTLY rather than as a result of the cell cycle progression delay, Second, Ectopic over-expression and genetic invalidation experiments have shown that Cyclin D1 promotes hepatocyte cell cycle progression. More importantly, Cyclin D1 overexpression restores the inhibitory effect of S6K deletion on S phase entry after (Partial HEPATOCOMY) PH, establishing a causal relationship between the mTOR/S6K pathway. The expression of Cyclin D, and cell cycle progression. Third, as demonstrated in several settings, Cyclin D1 is an early target following the temporary suppression of mTOR activity by pharmaceutical drugs. Interestingly, the mTOR pathway can control Cyclin D1 expression at the transcriptional and translational levels. (Espeillac et al.,2011).

CONCLUSIONS

Understanding the molecular mechanisms behind the mTOR pathway's control of the cell cycle is essential for all disease situations where mTOR inhibitor therapy is anticipated to be effective. From these results we concluded that the use of Rapamycin has been affected on checkpoints of cell cycle during embryogenesis, which appeared through its effects on cyclin D expression.

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