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Original Article

Sirolimus treatment induces dose-dependent involution of the thymus with elevated cellular respiration in BALB/c mice

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Abstract: Several *in vitro* and *in vivo* studies have shown that the mammalian target of rapamycin (mTOR) inhibitor sirolimus (rapamycin) suppresses thymus cellular respiration. The objective of this study is to investigate the chronic dose-dependent effects of sirolimus in the thymus. This was monitored using body weight, histomorphology, caspase-3 expression, cytochrome C immunohistochemistry, and cellular bioenergetics as surrogate biomarkers. BALB/c mice received intraperitoneal injections of either sirolimus (2.5, 5, or 10 μg/g) or dimethyl sulfoxide (0.1 μL/g) as a control for 4 weeks. At the end of the treatment, fragments were collected from the thymus, small intestine, adrenal gland, and kidney. They were processed for assessing histologic changes, measuring cellular respiration and ATP levels. Immunohistochemical stain of caspase-3 and cytochrome C was performed on paraffin-embedded tissue. The treated animals exhibited a dose-dependent reduction in weight gain despite adequate food intake. Sirolimus produced significant thymic derangements, manifested by dose-dependent tissue involution, increased cortical apoptotic bodies, increased caspase-3-positive lymphocytes, and increased rate of cellular respiration without a concomitant increase in cellular ATP. There were no similar changes in cellular ATP in the other assessed organs. The effects on thymic cellular bioenergetics suggest mitochondrial derangements, uncoupling of oxidative phosphorylation, and induction of apoptosis.

Keywords: Thymus, sirolimus, cellular respiration, cellular bioenergetics

Introduction

Mammalian target of rapamycin (mTOR), a highly conserved serine-threonine protein kinase, plays a crucial role in regulating protein transcription, translation, and cell growth by sensing intracellular and extracellular energy levels through phosphorylation [1, 2].

mTOR has a unique structure and belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family of protein kinases [3]. mTOR links to other proteins and serves as a core component of two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which regulate different cellular processes [4]. mTOR signaling is dysregulated in many diseases, including cancer; thus, mTOR has emerged as an excellent therapeutic target for innovative cancer treatment [5]. Inhibitors of mTOR, such as sirolimus (rapamycin), inhibit tumor growth by inducing tumor cell apoptosis and arresting tumor cell proliferation [6].

Sirolimus is an anti-tumor, anti-aging, and immunosuppressive agent that inhibits the proliferation of T and B cell populations [7]. Due to these immunosuppressive properties, sirolimus has been approved by the Food and Drug Administration (FDA) as a medication to prevent graft rejection after organ transplantation [8-10]. Studies have also shown that inhibiting mTOR promotes longevity and reduces age-related diseases [11]. Thus, regulating the

mTOR pathway is highly effective in mitigating many health risks.

Less information is known about its effects on processes such as cellular bioenergetics, glucose metabolism, and insulin resistance. Cellular bioenergetics encompasses the biochemical processes involved in energy conversion (biotransformation) [12]. Cellular respiration, on the other hand, describes the delivery of O_2 to the mitochondria, the breakdown of reduced metabolic fuels, the passage of electrons to O_2 to form H_2O (oxidation), and the resulting synthesis of adenosine triphosphate (ATP). Thus, impaired respiration implies any metabolic fuel abnormalities, including those involving glycolysis or mitochondrial function (e.g., mitochondrial derangements associated with apoptosis) [13, 14].

In vitro studies have also shown that inhibition of mTOR by the immunosuppressant sirolimus decreases the rate of cellular respiration [15-18]. These results are consistent with the evidence that mTOR influences the flux of nutrients into mitochondria [19, 20]. A recent study has also shown that sirolimus decreases the rate of thymic cellular respiration, and *in vivo* treatment of sirolimus in mice (2.5 μg/g/day) caused reversible thymic cortex involution due to induction of lymphocyte apoptosis [15-18]. Identifying the minimum dose of a drug with minimal side effects that will still achieve mTOR regulation is essential.

Therefore, we aimed to investigate the chronic dose-dependent effect of sirolimus on cellular bioenergetics in organs such as the thymus, small intestine, kidney, and adrenal gland in BALB/c mice. This effect was monitored using body weight, histomorphology, caspase-3 expression, and cytochrome C immunohistochemistry as surrogate biomarkers.

Materials and methods

Reagents and chemicals

Sirolimus was procured from MedChem Express LLC (Princeton, NJ, USA). The drug was dissolved in DMSO in a 50 μg/μL (55 mM), aliquot, and stored at -20°C, and diluted to 0.5 μg/μL in distilled water just before use. The Pd (II) complex of meso-tetra-(4-sulfonatophenyl)-tetra benzoporphyrin (Pd phosphor) was obtained from Porphyrin Products (Logan, UT, USA). The Pd phosphor was dissolved in distilled water and stored in small aliquots (one-time use) at -20°C. Anti-cleaved caspase-3 antibody and anti-cytochrome c antibody ([H-104]: sc-7159), both raised in rabbit, were procured from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Roswell Park Memorial Institute (RPMI) medium from Sigma-Aldrich (St. Louis, MO, USA) was used for all *ex vivo* studies.

Animals

Adult BALB/c mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). They were maintained at 22°C under 12-h light-dark cycles and provided with filtered water and standard rodent chow ad libitum. All studies involving animals were approved by the Animal Ethics Committee of the College of Medicine and Health Sciences, UAE University (ethical approval number: A29-13).

Experimental design and treatment

Mice received intraperitoneal injections of sirolimus (treated group) or Dimethyl sulfoxide (DMSO: control group) for 5 consecutive days each week for a total of 4 weeks. In this study, we used 3 different concentrations of Sirolimus based on its clinical dosage and its animal study dosage [21-23]. The Sirolimus doses used were 2.5 μg/g (5 μL/g), 5 μg/g (10 μL/g), and 10 μg/g (20 μL/g). For the control group, DMSO diluted to 1:100 with distilled water was used and administered to mice (actual DMSO dose was 0.1 μL/g). Several *in vivo* and *in vitro* studies have shown that the diluted concentration of DMSO used in this study was within the safe treatment range [24-27].

Fragments of the thymus, small intestine, adrenal gland, and kidney were obtained at the end of the *in vivo* treatment for assessing histologic changes and measuring cellular respiration and ATP levels as well as caspase-3 and cytochrome c expression.

Tissue collection and processing

The mice were anesthetized with 25% w/v urethane (10 L/g) administered by intraperitoneal route at the end of the experiment. The study organs were then carefully removed using a

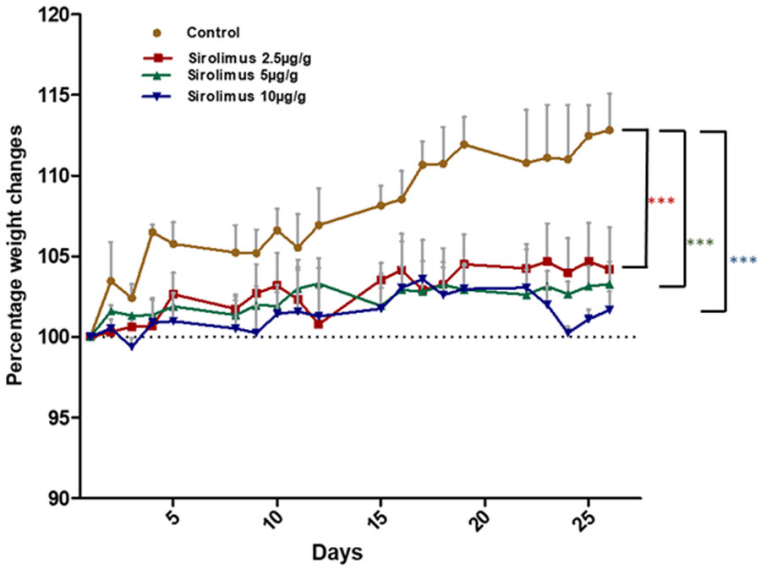

Figure 1. Weight changes in mice treated with DMSO (control) or sirolimus. The values are percentages of daily weight divided by the starting weight. Each data point represents the mean ± SD of 9 mice per group, pooled from 3 individual experiments. ***P<0.001.

sterile scalpel and immersed in ice-cold RPMI medium saturated with 95% oxygen and 5% carbon dioxide. A portion of the tissue was immediately immersed in ice-cold 2% trichloroacetic acid and processed for ATP determination, as previously described [15-17]. To determine the rate of cellular respiration at 37°C, another fragment of the tissue was immediately placed in an oxygen-measuring vial. A reaction mixture containing 1.0 mL of RPMI medium, 0.5% fat-free albumin, and 3 mM Pd phosphor was used for measuring cellular respiration [14-18].

Tissue preparation

For histologic analysis, tissue fragments were fixed in 10% formalin, dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 95%, 100%), cleared with xylene, and embedded in paraffin; 4-5 µm sections were cut and stained with Hematoxylin and Eosin (H&E). Additional 4-5 µm sections were obtained on silane-coated microscope slides to perform cytochrome c (1:750 dilution; Cell Signaling, USA) and caspase 3 (1:300 dilution; Cell Signaling, USA) immunostaining, as previously described. The mouse- and rabbit-specific horseradish peroxidase/3,3'-diaminobenzidine detection immunohistochemistry kit (Abcam, Cambridge, UK) was used for development after primary antibody incubation. On the basis of a semi-quantitative manual method, immunostaining intensity was scored as follows: strong (3+), moderate (2+), weak (1+), and negative (0). Grading was performed on the basis of percentage of positive cells [18-20, 28].

Statistical analysis

Results are expressed as either mean ± SD or mean ± SE where indicated. Statistical analysis was done with ordinary one-way ANOVA using Tukey's multiple comparison test. GraphPad Prism version 9 software program (San Diego, CA, USA) was used to perform statistical analysis. Ordinary two-way ANOVA was used to analyze multiple comparisons (**Figure 1**). Differences were considered significant when *P<0.05, **P<0.01, ***P<0.001, or ****P<0.0001.

Results

Effect of Sirolimus on body weight

The effect of Sirolimus or DMSO (control) administration on weight change in mice is shown in **Figure 1**. Sirolimus treatment diminished the weight gain in a dose-dependent manner. Consumption of food was then determined in two separate experiments to determine whether the diminished weight gain in Sirolimus treated group was due to reduced appetite or not. The average consumption of food for the control group was 5.7 g, for the 2.5 µg/g sirolimus group was 6.5 g, for the 5 µg/g sirolimus group was 6.4 g, and for the 10 µg/g sirolimus group was 6.2 g. The corresponding values in another experiment were 5.8 g, 6.2 g, 11.7 g, and 9.2 g, respectively.

Effect of Sirolimus on thymus weight

Next, we investigated whether sirolimus treatment affected the weight of the thymus, kid-

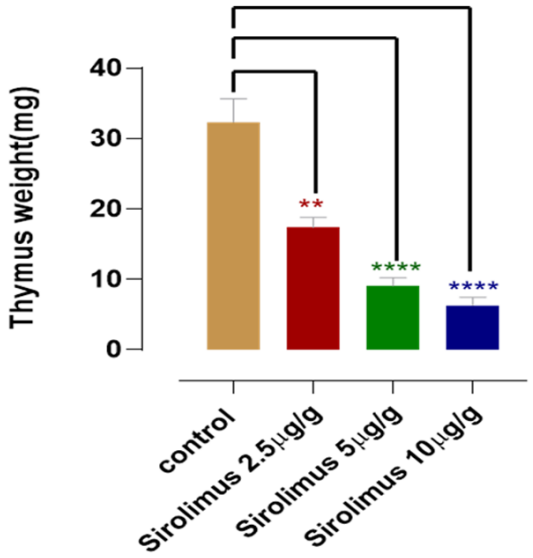

Figure 2. Thymus weight in mice treated with DMSO (control) or sirolimus. The data are expressed as means ± SEM of 3 replicates per group and are representative of at least three independent experiments. Asterisks denote significant differences in thymus weight in different sirolimus treatments compared to control mice $^{**}P<0.01$; $^{****}P<0.0001$.

neys, and adrenals. It was noted that sirolimus treatment produced a dose-dependent involution of the thymus (**Figure 2**).

As seen in **Table 1**, thymus weight in the control group was 31.6±7.3 g and 17.1±3.1 g (P=0.002) in the 2.5 μg/g sirolimus group, 8.7±2.6 g (P<0.0001) in the 5 μg/g sirolimus group, and 7.7±3.1 g (P<0.0001) in the 10 μg/g sirolimus group. On the other hand, the weights of the adrenal glands and kidneys remained unchanged (**Table 1**).

Effect of Sirolimus on thymus cellular respiration

The effects of chronic sirolimus treatment on cellular respiration in the studied organs (thymus, kidney, small intestine, and adrenal gland) were assessed. Representative measurements of mitochondrial respiration for all the studied organs are shown in **Figure 3**, and a summary of the results is shown in **Table 2**.

In mice treated with sirolimus, the rate of thymus cellular respiration was significantly increased in a dose-dependent manner (P≤ 0.026). These changes in cellular bioenergetics were not observed in the other organs studied (**Table 2A**). Thus, it appears that *in vivo* sirolimus treatment induces dose-dependent impairments only in thymus cellular respiration. Cellular ATP levels were also measured in the four organs studied but ATP contents did not differ significantly between the groups in any of the organs (**Table 2B**).

Effect of sirolimus on histology of thymus and other organs

Thymus, kidney, small intestine, and adrenal gland tissues were excised from animals of control and sirolimus-treated groups and subjected to histologic examination with H&E staining. Immunohistochemical staining for caspase-3 and cytochrome-3 proteins was also performed. The tissue structure was examined, and representative images are shown in **Figure 4**.

The thymic structure in the mice treated with sirolimus showed a loss of well-characterized demarcation between the thymic cortex and medulla with involution of the thymic cortex. Increased cortical apoptotic bodies were also observed. There were 8-12 apoptotic bodies per high-power field (HPF) at 10 μg/g sirolimus, 3-6 apoptotic bodies per HPF at 5 μg/g, 3 apoptotic bodies per HPF at 2.5 μg/g, and no detectable apoptotic bodies in the thymic cortex in the control group. Kidney sections demonstrated variable degrees of acute tubular injury. Renal morphologic changes included flattening of the proximal tubular epithelium, loss of brush border, focal denudation of tubular basement membranes, focal luminal necrotic cellular debris, and mild interstitial edema. Baseline renal tubular changes were identified in the control group, reflecting a prolonged ischemia. However, additional morphologic deterioration of renal tubules was noticeable with higher sirolimus doses of 5 and 10 μg/g. These renal changes were similar in the three doses studied. In contrast, the structures of the small intestine and adrenal gland remained intact.

Sirolimus treatment was associated with dose-dependent increases in caspase-3-positive cortical lymphocytes in the thymus (e.g., 10 per HPF at 10 μg/g sirolimus vs. ≤5 per HPF in the control group) (**Figure 5**). The increase in cytochrome C-positive cortical lymphocytes was

Table 1. Effects of sirolimus on size of the studied organs

	Control (n=6)	Sirolimus (n=6)		
		2.5 μg/g	5 μg/g	10 μg/g
Thymus	31.6±7.3	17.1±3.1**	8.7±2.6****	7.7±3.1****
Adrenal gland	8.4±0.5	9.2±0.7	7.4±1.4	9.3±0.7
Kidney	336.0±27.0	349.0±69.0	334.0±74.0	343.0±83.0

**P<0.01 or ****P<0.0001 (compared to the control group). Values (in mg) are mean ± SEM.

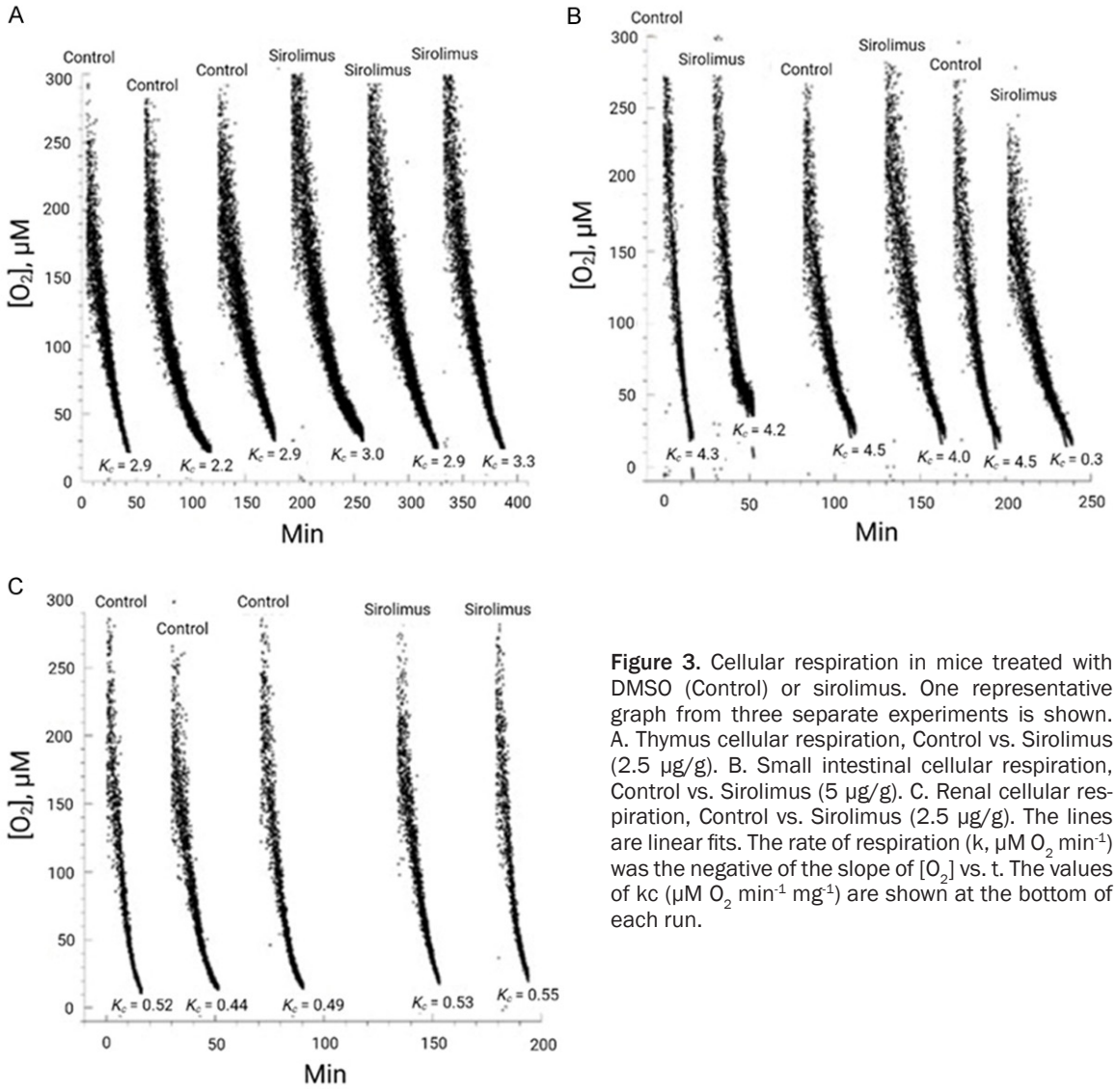

Figure 3. Cellular respiration in mice treated with DMSO (Control) or sirolimus. One representative graph from three separate experiments is shown. A. Thymus cellular respiration, Control vs. Sirolimus (2.5 μg/g). B. Small intestinal cellular respiration, Control vs. Sirolimus (5 μg/g). C. Renal cellular respiration, Control vs. Sirolimus (2.5 μg/g). The lines are linear fits. The rate of respiration (k, μM O_2 min^{-1}) was the negative of the slope of [O_2] vs. t. The values of kc (μM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run.

minimal, with most fields revealing an absence of, or only a few positive cells (**Figure 5**).

Discussion

Advances have been made over the years in understanding the mTOR pathway, which is involved in detecting environmental conditions and controlling most aspects of metabolism at both cellular and organismal levels [29]. Since the pathway plays a crucial role in maintaining cellular and physiological homeostasis, dysregulation of it signaling has shown to have implications for metabolic disorders, cancer, neurodegeneration, and aging [30].

The mTOR inhibitors sirolimus and everolimus are FDA-approved drugs for treating cancer in

Table 2. Effects of sirolimus on tissue cellular bioenergetics in the studied tissues. Values are mean ± SD (n)

	Control (n)	Sirolimus (n)		
		2.5 μg/g	5 μg/g	10 μg/g
A.	*Cellular Respiration (μM O_2 mg^{-1} min^{-1})*			
Thymus	0.26±0.03 (6)	0.30±0.02 (6)[a]	0.38±0.06 (6)[b]	0.50±0.05 (6)[c]
Small intestine	0.47±0.11 (9)	0.36±0.07 (4)	0.39±0.04 (9)[d]	0.32±0.04 (2)
Adrenal gland	0.62±0.03 (2)	0.54±0.16 (3)	0.32±0.28 (3)	0.45±0.03 (3)
Kidney	0.48±0.03 (6)	0.49±0.08 (3)	0.42±0.07 (3)	0.52±0.03 (3)
B.	*Cellular ATP (pmol mg^{-1})*			
Thymus	5,110±2,304 (7)	4,118±1,763 (8)	4,058±2,152 (9)	4,071±1,92 (7)
Small intestine	1,045±248 (3)	2,259±874 (3)	1,715±111 (3)	1,765±790 (3)
Adrenal gland	2,414±300 (3)	2,307±578 (3)	2,515±746 (3)	2,410±325 (3)
Kidney	1,523±448 (3)	1,648±371 (3)	1,834±634 (3)	1,981±953 (3)

(a) P=0.026 (Control vs. 2.5 μg/g sirolimus); (b) P=0.002 (Control vs. 5 μg/g sirolimus); (c) P=0.002 (Control vs. 10 μg/g sirolimus); (d) P=0.019 (Control vs. 5 μg/g sirolimus).

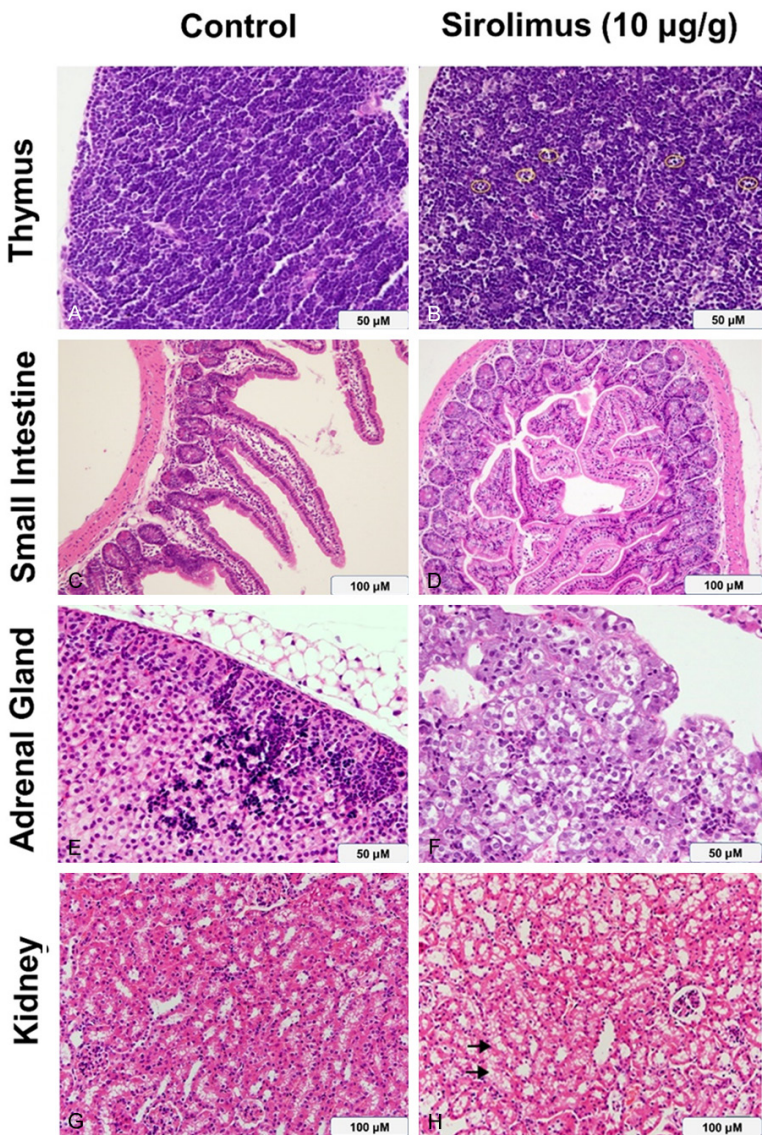

Figure 4. Representative histology (H&E) of thymus, small intestine, adrenal gland, and kidney from control group and sirolimus (10 μg/g) group are shown for comparison. A. Thymic tissue from control mice. B. Thymic tissue in the sirolimus group reveals a marked increase in apoptotic bodies (circles) in comparison to control group (12 apoptotic bodies per HPF in comparison to no detectable apoptotic bodies in the control group). This was associated with loss of the normal demarcation between cortex and medulla (image not highlighted here). C. Small intestine from control mice. D. Small intestine from sirolimus-treated mice with no histologic changes; E. Adrenal tissue from control mice. F. Adrenal tissue from sirolimus-treated mice; adrenal structures remained intact with no morphological changes noted. G. Renal sections from control mice. H. Renal section from sirolimus-treated mice, demonstrating focal tubular epithelial vacuolization (arrows), disruption of intercellular connections and cellular membrane with preserved glomeruli in the sirolimus group. Tubular epithelial outlines are preserved in the control group (G).

humans [31]. In addition, sirolimus and its analogs have been used as immunosuppressants for the prevention

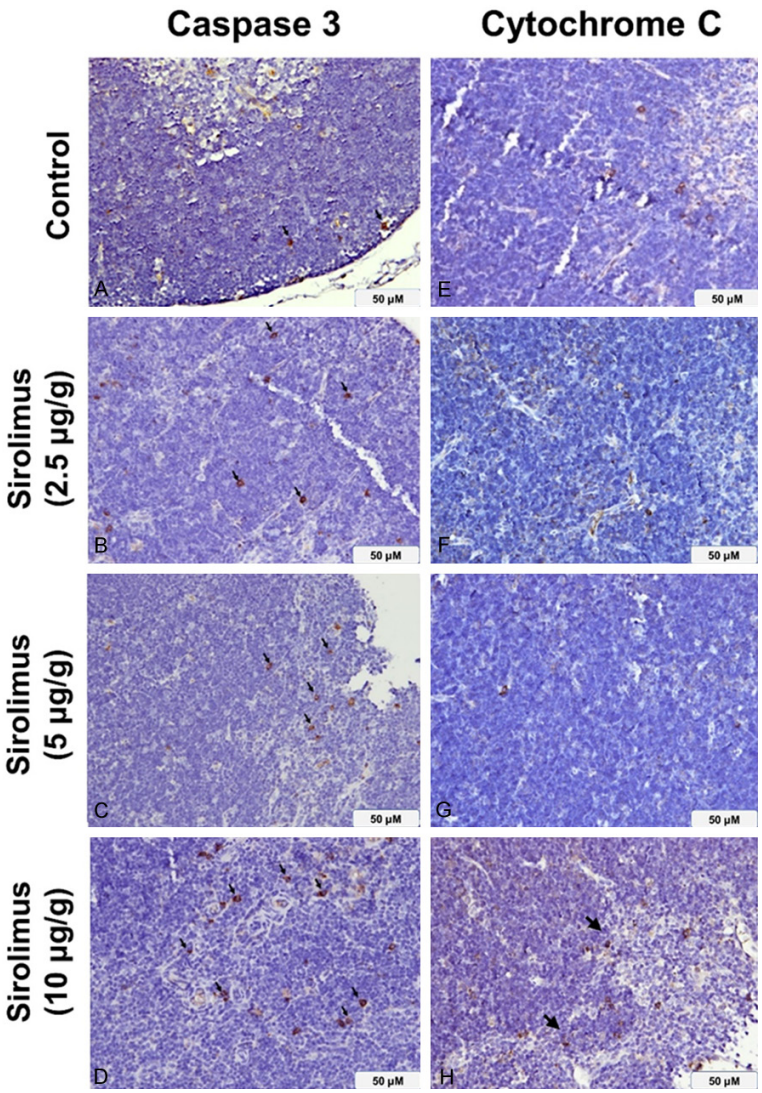

Figure 5. Immunohistochemical staining of mouse thymus tissue with caspase 3 and cytochrome c. Sirolimus treatment was associated with a dose-dependent increase in caspase 3 positive cortical lymphocytes. Caspase-3 stained (A) control mouse thymus tissue, which shows no detectable positive cells. (B) Thymic tissue from mouse treated with sirolimus 2.5 µg/g shows positivity for 3-4 apoptotic lymphocytes. (C) Thymic tissue from mouse that received sirolimus 5 µg/g, shows positivity for 4-5 apoptotic bodies. (D) Thymus from mouse which received the highest Sirolimus dose (10 µg/g) has 10-12 positive cortical lymphocytes. Cytochrome c staining on (E) control thymus tissue, mice treated with (F) sirolimus dose of 2.5 µg/g, (G) sirolimus dose of 5 µg/g, and (H) sirolimus dose of 10 µg/g. All the studied groups show minimal changes in the number of cytochrome c positive cortical lymphocytes.

of graft rejection for over 20 years [32]. There are many benefits of using mTOR receptors as therapeutic agents; however, some studies have found harmful side effects in mice experiments, such as glucose intolerance, insulin resistance, prostate regeneration, increase in cataract severity, and nephrotoxicity [33]. Most side effects can be mitigated by determining the minimum dose of a drug with the correct dosing regimen. The results of one study suggest that intermittent rather than continuous dosing can alleviate many of the negative effects of sirolimus treatment [34].

We previously examined the *ex vivo* and acute *in vivo* effects of sirolimus treatment over a period of 4 days on thymic tissue. An involution of the thymus was observed after just 4 days of treatment, and it returned to its normal size 10 days after ceasing the treatment. In this follow up study, we aimed to determine the effect of sirolimus over a longer period. Additionally, varying sirolimus concentrations were tested because mice seem to react differently to them. Four different organs were selected for the study: the thymus, kidney, small intestine, and adrenal glands to determine the effects of chronic sirolimus treatment.

One of the key findings from the present study is that chronic sirolimus treatment significantly reduced weight gain compared to control mice. A reduction in the percentage of weight gain was observed at all three concentrations of sirolimus dosing and was independent of food intake. Since mTOR is known to influence the flux of nutrients into the mitochondria [19, 20], we next investigated whether sirolimus treatment impaired cellular bioenergetics, which includes cellular respiration and quantification of ATP in the tissues studied (the thymus, small intestine, adrenal gland, and kidney). Three separate experiments (three mice per group for each experiment, 36 mice total) were performed. In mice treated

with sirolimus, the rate of thymic cellular respiration was significantly increased in a dose-dependent manner without a concomitant increase in thymus cellular ATP. These results suggest that uncoupling oxidative phosphorylation is responsible for the increased rate of oxidation within the mitochondria. These changes in cellular bioenergetics were not observed in the other organs studied. Thus, it appears that *in vivo* sirolimus treatment induces dose-dependent impairments in thymic cellular bioenergetics. This finding suggests that altered cellular metabolism (as expected by blocking mTOR) might be responsible for the observed weight profiles [19, 20].

This chronic *in vivo* study also examined the thymic pathology, such as size, histology, and expression of active caspase-3 and released cytochrome C in mice treated with three different sirolimus dosing regimens. The treatment was administered for 5 consecutive days for 4 weeks. Sirolimus treatment resulted in thymic involution and induction of lymphocyte apoptosis. Sirolimus (5 and 10 μg/g) caused mild morphologic deterioration of the renal tubules in mice. However, we did not observe any morphologic changes at 2.5 μg/g. This variability in response has been reported previously at similar doses [35]. These findings were tissue-specific and were observed only in the thymus and kidney.

These results are different from previously reported *in vitro* findings, which showed decreased cellular respiration in the presence of sirolimus [15-18]. Thus, it is likely that the *in vivo* effects of sirolimus on thymic cellular respiration represent mitochondrial derangements (uncoupling oxidative phosphorylation due to activation of the mitochondrial cell death pathway) associated with apoptosis. These results are consistent with the known role of mTOR signaling in cellular metabolism and the induction of lymphocyte apoptosis [13-15]. The immunosuppressive effects of rapamycin are mainly due to its ability to block T cell activation, which is an important aspect of the adaptive immune response. The results also confirm the effect of sirolimus on T cell development in mice, leading to major thymic atrophy [36-38].

Although this study answers some aspects of cellular bioenergetics after chronic sirolimus treatment, the main mechanism behind thymus involution, reduced weight gain, and inhibition of T and B lymphocyte proliferation needs to be elucidated. Future studies will focus on expression analysis for different gene markers and the complex role of BMP and other signaling markers in energy metabolism.

The findings presented here have implications for better understanding on how metabolic diseases and cancer can alter these crucial pathways.

Conclusion

Sirolimus treatment induces thymic involution associated with cortical lymphocyte apoptosis and cellular bioenergetic disturbances. These effects were tissue specific and dose-dependent. Sirolimus treatment also causes systemic metabolic derangements, such as reduced weight gain. The current findings warrant further detailed investigations to assess how these molecular events affect the clinical use of sirolimus.

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Disclosure of conflict of interest

None.

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