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Rapamycin Does Not Act as a Dietary Restriction Mimetic in the Protection against Ischemia Reperfusion Injury

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Kidney transplantation · Ischemia-reperfusion injury · Rapamycin

Abstract

Introduction: Short-term fasting protects against renal ischemia reperfusion injury (IRI). mTOR signaling is downregulated and may be involved in its protective effect. Rapamycin is considered a possible mimetic as it inhibits the mTOR pathway. This study examines the effect of rapamycin on renal IRI. ***Material and Methods:*** Mice were divided into four groups: ad libitum (AL), fasted (F), AL treated with rapamycin (AL+R), and F treated with rapamycin (F+R). Rapamycin was administered intraperitoneally 24 h before bilateral renal IRI was induced. Survival was monitored for 7 days. Renal cell death, regeneration, and mTOR activity were determined 48 h after reperfusion. Oxidative stress resistance of human renal proximal tubular and human primary tubular epithelial cells after rapamycin treatment was determined. ***Results:*** All F and F+R mice survived the experiment. Although rapamycin substantially downregulated mTOR activity, survival in the AL+R group was similar to AL (10%). Renal regeneration was significantly reduced in AL+R but not in F+R. After IRI (48 h), pS6K/S6K ratio was lower in F, F+R, and AL+R groups compared to AL fed animals ($p = 0.02$). In vitro, rapamycin also significantly downregulated mTOR activity ($p < 0.001$) but did not protect against oxidative stress. ***Conclusion:*** Rapamycin pretreatment does not protect against renal IRI. Thus, protection against renal IRI by fasting is not exclusively mediated through inhibition of mTOR activity but may involve preservation of regenerative mechanisms despite mTOR downregulation. Therefore, rapamycin cannot be used as a dietary mimetic to protect against renal IRI.

Introduction

Dietary restriction (DR), defined as reduced food intake without causing malnutrition, can be applied by short-term fasting or calorie restriction for a longer period. DR has been reported to extend lifespan in several organisms, from rodents [1] to other life forms including nonhuman primates [2]. Furthermore, animals undergoing DR have increased resistance to different forms of stress [3, 4]. In humans, the effects of DR on health and lifespan are difficult to determine, although long-term DR seems to have a favorable impact on age-related morbidity including cardiovascular fitness, BMI, and insulin sensitivity [5–7]. Ischemia reperfusion injury (IRI) is still an inevitable problem during kidney transplantation,

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resulting in oxidative damage by production of reactive oxygen species. Especially proximal tubular cells are prone to oxidative stress. Previously, we showed that both short-term fasting (water only diet) and 14 days of 30% calorie restriction protect against renal IRI in C57BL/6 mice, improving both kidney function and survival [8]. In humans, we showed that DR improved outcome after kidney donation and transplantation and may improve the therapeutic window of toxic anticancer agents [9, 10]. Although we showed that DR can attenuate renal IRI, it remains to be elucidated which underlying mechanisms cause these protective effects. Furthermore, applying fasting or any form of DR to humans before surgery is still considered unwanted. A DR mimetic, with similar effects as DR, would be an alternative method to attenuate renal IRI in kidney transplantation. The target of rapamycin (TOR) pathway is a nutrisensing pathway which is known for its longevity effects in *C. elegans* and *Drosophila* when suppressed [9, 10]. Rapamycin, an mTOR inhibitor, has been shown to have similar effects as DR in inducing increased lifespan in yeast and in mice [11–13]. Since downregulation of mTOR activity is a molecular hallmark of fasting and is believed to be involved in its protective effect, rapamycin could act as a DR mimetic and may provide protection against IRI by downregulating mTOR signaling. This study was designed to assess the effects of rapamycin on in vitro-induced oxidative stress and to examine its potential as a DR mimetic to attenuate renal IRI in mice.

Materials and Methods

In vivo

Male C57BL/6 mice (~25 g) were obtained from Harlan (Horst, The Netherlands). On arrival, mice were placed with 3–4 mice in individually ventilated cages and housed under standard circumstances. All mice had free access to water. All mice acclimated for 7 days before start of the experiment. Fasted mice had free access to water but no access to food for 3 days prior to IRI. Animal experiments were approved by the University Animal Experiments Committee (Protocol No. 105-12-13) under the Dutch National Experiments on Animals Act, compiled with Directive 86/609/EC (1986) of the Council of Europe.

Rapamycin

24 h before IRI, all mice received an intraperitoneal injection of 0.2 mL with either 5 mg/kg rapamycin (LC Laboratories) dissolved in dimethylsulfoxide (Sigma-Aldrich) and diluted in phosphate-buffered saline (PBS) or vehicle of dimethylsulfoxide diluted with PBS.

Surgical Procedure

Animals were anaesthetized with isoflurane 4% (Pharmachemie BV) in O_2 and placed on heated plates. Through midline incision, both kidneys were localized and renal pedicles were dissected. A vascular clamp was placed for 37 min to induce bilateral renal ischemia. After the clamps were removed, reperfusion of the kidneys was visually assessed and the abdomen was closed with 5/0 Vicryl sutures. Animals were monitored until they regained consciousness before moving them from the operating room to the stable. At 48 h and 7 days after reperfusion, animals were exsanguinated through heart puncture under anesthesia. Kidneys were collected and immediately stored in either liquid nitrogen or 4% formaldehyde.

Western Blot

mTOR. Kidney tissue was supplemented with 300 µL Laemmli buffer and 100 mM dithiothreitol and heated for 5 min at 95°C. 25 µL is loaded onto 9% SDS-PAGE gel with protein size standards (Precision Plus Protein™ Standards, Bio-Rad Laboratories B.V.). After electrophoresis (100 V, ~1.5–2.5 h), proteins were transferred to Immobilon-FL PVDF membranes (Millipore B.V.) for 1 h at 250 mA. Membranes were blocked by incubation with Odyssey blocking buffer (Li-COR Biosciences GmbH) and washed with TBS/Tween 0.1%. Primary antibody incubations against p-S6-kinase or S6-kinase (Cell Signaling, dilution 1:1,000) and β-actin (dilution 1:20,000) (Sigma-Aldrich) were left overnight at 4°C. After washing with TBS/Tween 0.1%, membranes were incubated for 1 h with IRDye® 800CW conjugated goat anti-mouse IgG (H+L) and IRDye® 700DX conjugated goat (polyclonal) anti-mouse IgG (H+L) antibodies (dilutions 1:5,000, both from LI-COR Biosciences GmbH). Antibody binding was detected using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences GmbH) and analyzed using the LI-COR Odyssey infrared imaging system application software (v3.0).

Apoptosis. To determine apoptosis, the protein levels of PARP/cleaved PARP (Cell Signaling) were determined using 6% SDS-PAGE gel and the primary antibody PARP (1:1,000).

Proliferation. For PCNA, a 12% SDS-PAGE gel and the primary antibody PCNA (Abcam, UK), dilution 1:1,000, were used. All data were normalized to β-actin.

In vitro

Cell Cultures

Human renal proximal tubular (HK-2) cells and human primary tubular epithelial cells (PTECs) were cultured in Dulbecco's Modified Eagle's Medium-F12 medium (Lonza) supplemented with 1% penicillin/streptomycin (Gibco), ITS 100 µL/mL (Lonza), hydrocortisone 100 µL/mL (Sigma-Aldrich), EGF 100 µL/mL (Sigma-Aldrich), and tri-iodothyroxine 100 µL/mL (Sigma-Aldrich). 1% fetal bovine serum (Lonza) was added to the medium of PTECs. The cells were grown at 37°C in a humidified 5% CO_2 and 95% O_2 atmosphere. Medium was changed every 2–3 days. Cells were cultured in 25–75 cm^2 flasks and passaged when 80% confluence was reached.

Inducing Oxidative Stress and Rapamycin Treatment

Hypoxia/reoxygenation. Hypoxia was induced by bags of Anaerocult A®. Cells were left in 0.1% O_2 for 72 h. Cells were stressed by placing them in a humidified chamber with room air and 5% CO_2 for 24, 48, or 72 h.

H_2O_2. H_2O_2 30% (Merck) was freshly prepared before use and was diluted to 3 mM with medium before application. The cells were incubated 2 h with 3 mM H_2O_2.

Rapamycin. Cells were treated with rapamycin (10 ng/mL), dissolved in dimethylsulfoxide, and diluted in medium either 24 h before adding H_2O_2 or directly before placement in 0.1% O_2.

Western Blot

The protein expression levels of mTOR were determined by Western blot. 15×10^6 cells were used and blotted as described before.

Cell Viability

Cell viability was measured using a XTT assay. 10,000 cells/well were plated in a 48-well plate and incubated for 72 h before stress. XTT (sodium 2,3-bis-[2-methoxyl-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma-Aldrich) was measured at $t = 0$, 24, 48, and 72 h after oxidative stress. 250 μL of XTT (1 mg/mL) and 25 mM PMS (phenazine methosulfate, Sigma-Aldrich) were added and incubated at 37°C for 1 h. Absorbance of aliquots from each well was read by Wallac-Victor 1420 plate reader.

Cell Death

Cells were stressed using H_2O_2 as described above. 120,000 cells were plated/well in a 6-well plate. 10 μL trypan blue (Sigma-Aldrich) was added to 10 μL cell suspension, and cell death was determined using a Bürker counter.

Apoptosis

For the in vitro apoptotic assay, 1×10^6 HK-2 cells were plated in a 96-well plate. Cells were stressed with H_2O_2 as described before. Cells were stained with the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™). Cells were washed with PBS and resuspended in 1X binding buffer. 100 μL of the solution was transferred to a 5-mL tube. Then, 5 μL PE annexin V and 5 μL 7-AAD were added and incubated for 15 min at room temperature in the dark. After incubation, 400 μL of binding buffer was added and after, cells were acquired on FACS LSRII™ flow cytometer (BD Biosciences). A minimum of 20,000 live events were acquired based on forward and side scatter. Data analysis was performed using FlowJo™ (Tree Star) with PE channel on x-axis and PE-Cy5 on y-axis. The plots were dissected into three populations by placing a quadrant. Cells negative for both annexin V and 7-AAD were considered live non-apoptotic cells. Cells which were positive for annexin V and negative for 7-AAD were the cells undergoing apoptosis (early apoptosis), while the cells positive for both annexin-V and 7-AAD were considered late apoptotic or necrotic cells.

Statistical Analysis

All data are expressed as means ± SEM. Significance was tested with Student's *t* test using SPSS, version 20.0 (IBM Corp.). *p* values <0.05 were considered significant.

Results

In vivo

Rapamycin Pretreatment Does Not Protect against Renal IRI

As expected, survival after renal IRI in fasted animals was 100% at postoperative day 7. Fasting + rapamycin gave a similar survival rate (100%). There was no difference in survival between vehicle-treated controls and rapamycin-treated animals. Ninety percent of the animals died or had to be killed because of morbidity associated with irreversible kidney failure within 5 days after IRI (Fig. 1a).

mTOR Activity Decreases after Rapamycin Treatment

Twenty-four hours after intraperitoneal injection with rapamycin, pS6K/S6K ratio was measured to determine mTOR activity. In AL mice treated with rapamycin and fasted animals treated with rapamycin, the pS6K/S6K ratio was significantly lower compared to vehicle-treated mice ($p = 0.01$ and $p = 0.048$, respectively). Fasted mice showed a similar decrease in mTOR activity. Forty-eight hours after reperfusion, pS6K/S6K ratios were significantly higher in AL mice compared to all other groups ($p = 0.03$, $p = 0.01$, $p = 0.002$, rapamycin-treated animals, fasted, and fasted with rapamycin treatment, respectively) (Fig. 1b).

Cellular proliferation is inhibited after renal IRI in mice treated with rapamycin. Proliferation is needed after renal IRI to replace apoptotic cells by healthy cells. PCNA is used as a marker for proliferation and cell repair. Forty-eight hours after reperfusion, PCNA levels in kidney tissue lysates were significantly higher in fasted animals ($p = <0.001$) compared to rapamycin-treated and control animals (Fig. 1c).

Rapamycin Does Not Reduce Apoptosis after IRI

PARP is cleaved during the apoptotic cascade and is therefore used as a biomarker for apoptosis. At $t = 0$, all fasted animals (both with and without rapamycin) had significantly higher cleaved PARP/PARP ratio ($p < 0.05$). At 48 h after reperfusion, this ratio was significantly lower in fasted animals ($p < 0.001$, $p < 0.001$, and $p = 0.04$ compared to AL, F+, and AL+R, respectively). In fasted mice treated with rapamycin, this effect was diminished, resulting in significantly higher cleaved PARP/PARP ratio, compared to fasted animals. In AL mice, there was no difference between rapamycin- or vehicle-treated mice and no difference with fasted and rapamycin-treated mice (Fig. 1d).

In vitro

In mice, rapamycin did not protect against IRI. The pathophysiology of IRI, however, is complex, involving oxidative stress and a subsequent immunological response, which could cause even more cell damage. Therefore, we investigated the effect of rapamycin on oxidative stress resistance per se in two renal cell lines.

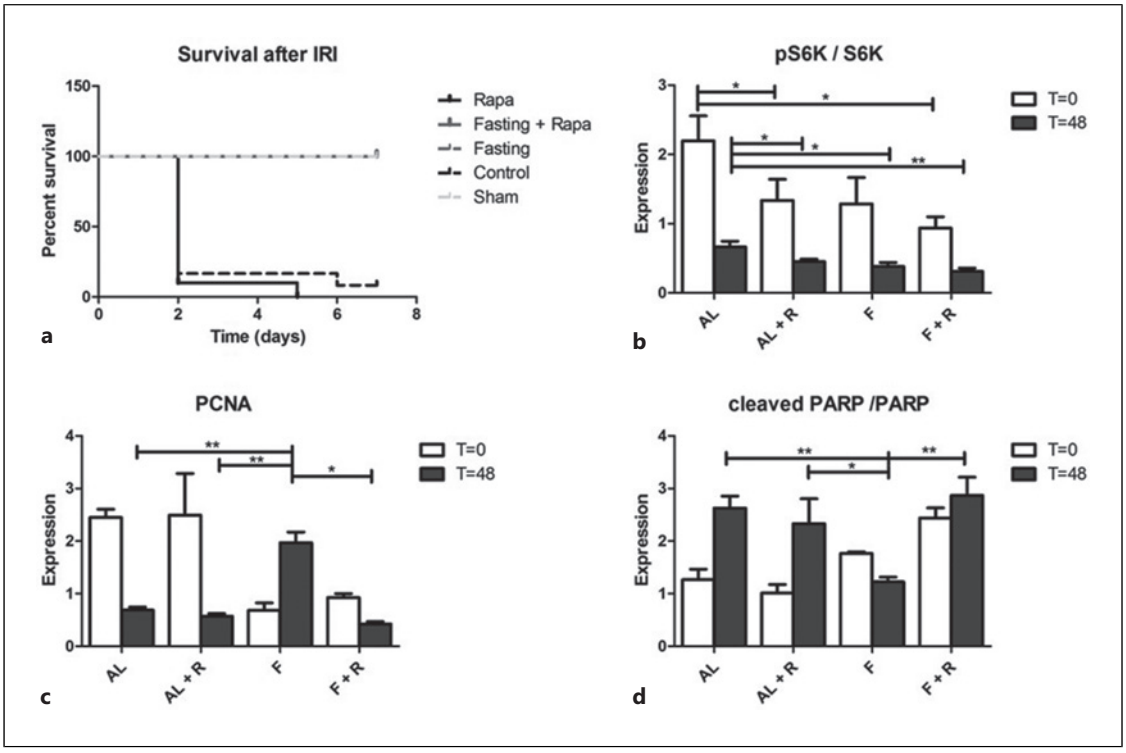

Fig. 1. Rapamycin treatment does not protect against renal IRI. **a** Improved survival after renal IRI by 3 days of preoperative fasting and fasting combined with rapamycin treatment but not rapamycin pretreatment alone. **b** mTOR activity is downregulated in the kidney after fasting and rapamycin treatment. **c** Tubular proliferation after renal IRI is increased in fasted mice but not in rapamycin-treated animals. **d** Fasting, but not rapamycin treatment, reduces levels of apoptosis after renal IRI. $T = 0$: levels in kidney tissue after fasting and/or rapamycin treatment, before induction of renal IRI. $T = 48$: 48 h after induction of IRI. $*p < 0.05$ $**p < 0.01$. AL, ad libitum fed animals; AL+R, ad libitum fed animals with rapamycin treatment; F, fasted animals; F+R, fasted animals with rapamycin treatment.

Rapamycin Does Not Protect against Oxidative Stress in vitro

Rapamycin-treated cells had similar growth curves as control cells, although rapamycin seemed to delay proliferation. H_2O_2 is a robust stressor in both HK-2 cells and PTECs, resulting in reduced proliferation up to 72 h. In PTECs, regeneration was seen after 48 h, when cells started to proliferate again. Hypoxia/reoxygenation had a similar, although less distinctive, effect (Fig. 2). When cells were incubated with rapamycin before oxidative stress, a significant reduction in proliferation was seen compared to controls. Rapamycin did not improve proliferation after oxidative stress, compared to controls.

In HK-2 cells, no difference in cell death was seen between rapamycin and controls. Hypoxia/reoxygenation resulted in significantly more cell death at 24 h but not at 48 and 72 h (Fig. 3). Oxidative stress in rapamycin-treated cells resulted in significantly more cell death compared to controls and to rapamycin-treated cells without stress. Cell death was equal between the two stressed groups (Fig. 3). In both HK-2 and PTEC, there was no difference in the percentage of dead cells after 1 h of incubation with H_2O_2 between rapamycin-treated cells and control cells (Fig. 3). After staining with annexin V, no significant differences were found in percentage of apoptotic cells or necrotic cells when stressed HK-2 cells were compared to stressed HK-2 cells pretreated with rapamycin (Fig. 4).

Discussion

Both DR and mTOR inhibition by rapamycin are associated with prolonged longevity in multiple species [1, 2, 11, 12]. DR, like short-term fasting, induces robust protection against renal IRI. Although preoperative overnight fasting is practiced to prevent aspiration of stomach content, the period is not sufficient to induce a protective response as seen after 3 days of fasting in mice.

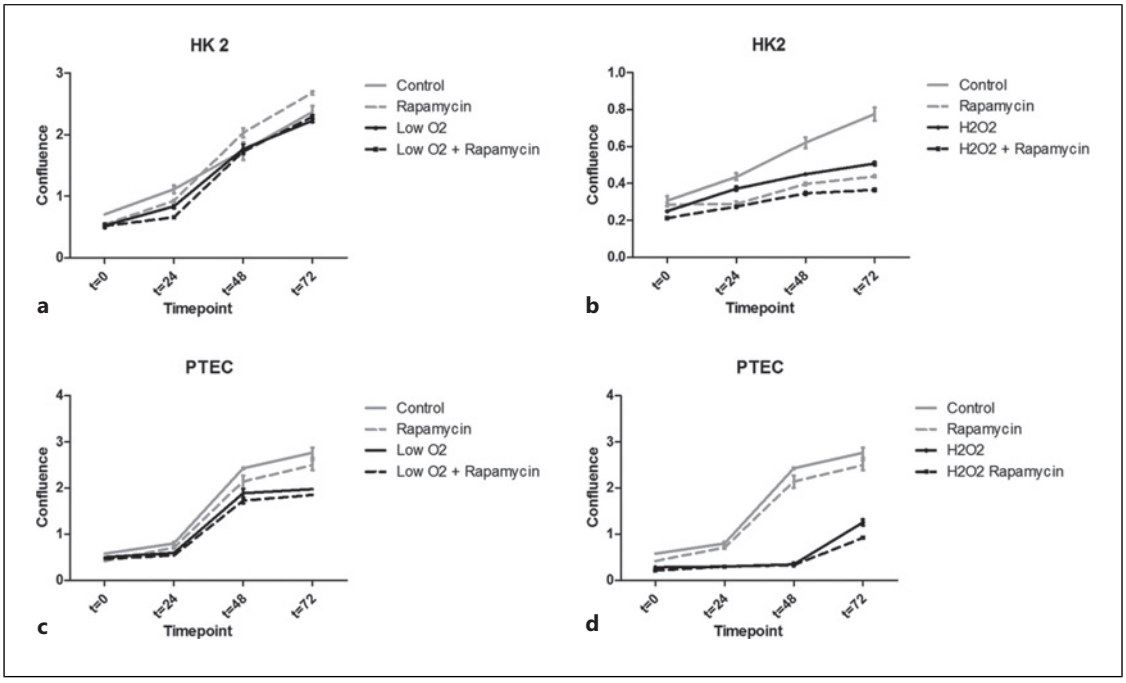

Fig. 2. Rapamycin does not increase cell viability in renal cells. Proliferation in HK 2 cells (**a**, **b**) and PTEC (**c**, **d**). Both hypoxia and reoxygenation (**a**, **c**) and H_2O_2 treatment (**b**, **d**) resulted in reduced cellular proliferation. $t = 0$: before induction of oxidative stress; t = 24, 48, and 72: 24, 48, and 72 h after induction of oxidative stress.

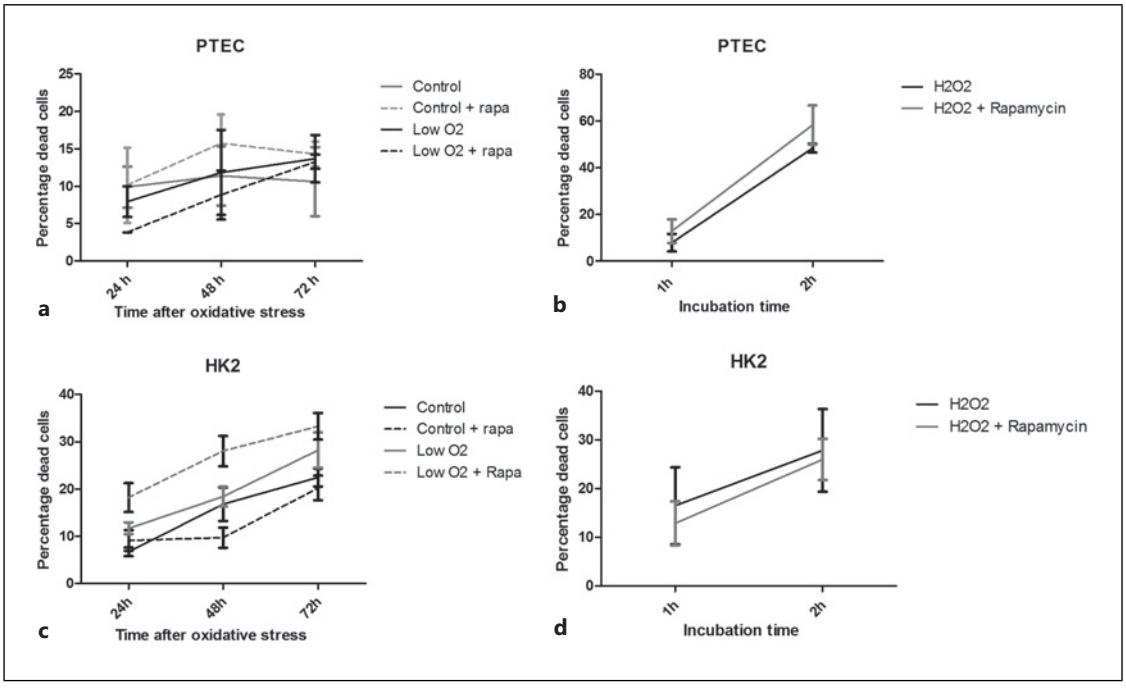

Fig. 3. Rapamycin does not protect against oxidative damage-induced cell death. Percentage of dead cells after oxidative stress in HK-2 cells and PTEC. In both cell lines, rapamycin treatment did not protect against oxidative stress induced by hypoxia and reoxygenation (**a**, **c)** or H_2O_2 treatment (**a**, **d**).

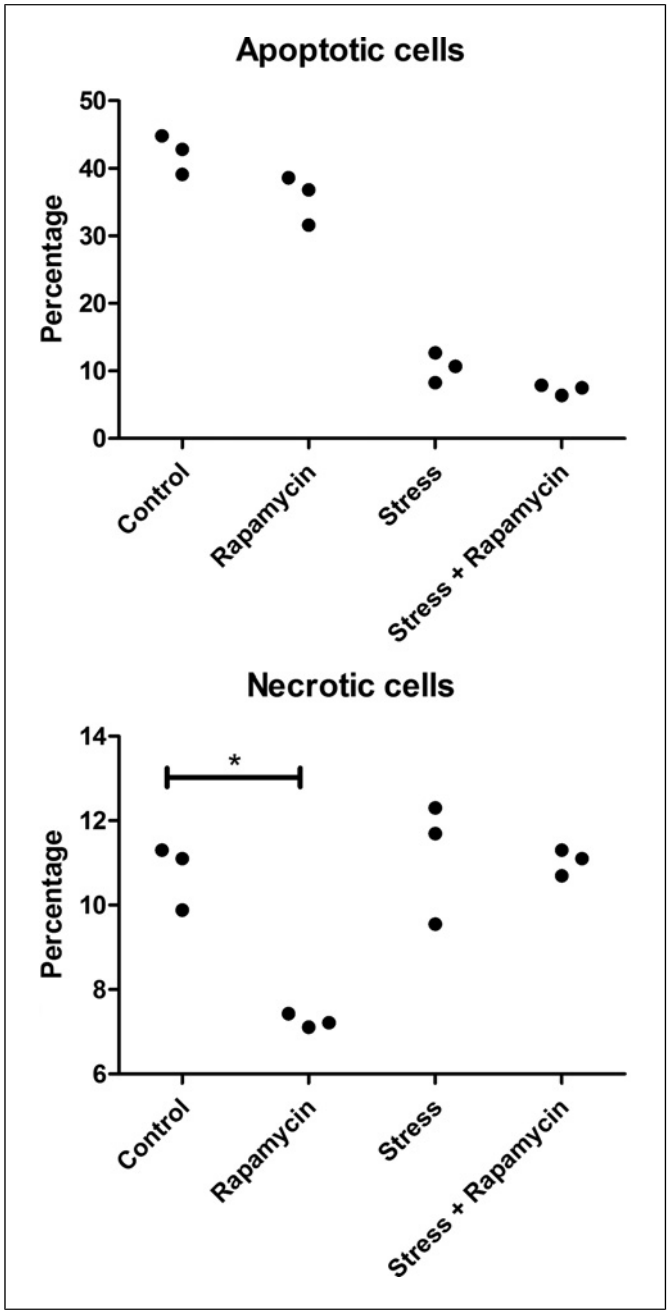

Fig. 4. Rapamycin does not protect against oxidative damage-induced apoptosis. Percentage apoptotic cells/necrotic cells based on annexin V staining. There is no significant difference between the group stressed by H_2O_2 and the stressed group pretreated with rapamycin. *$p < 0.05$.

Our hypothesis was that rapamycin could act as a DR mimetic, inducing similar protection against IRI. Unfortunately, our study failed to prove this hypothesis. Rapamycin targets mTOR, an atypical serine/threonine protein kinase which forms two distinct complexes (mTORC1 and mTORC2) by interaction with several proteins. mTORC2 is downregulated after continuous administration of rapamycin [14]. One dose of rapamycin, as we administered, is therefore likely to only downregulate mTORC1 [15]. Most of the functions of mTOR are attributed to mTORC1, and less is known about mTORC2. mTORC1 activity is regulated through multiple cellular processes, like cell growth, cellular stress, energy status, oxygen consumption, and amino acid metabolism [14].

mTORC1 activation leads to protein and lipid synthesis in proliferating cells and activates metabolism and ATP production [14, 16]. Likewise, inhibition of mTOR activity by DR decreases cellular proliferation and metabolism. Furthermore, mTOR inhibition stimulates autophagy, a process where cells are broken down and damaged organelles can be recycled, which is needed during nutritional deprivation [17]. Autophagy is implied as an obligatory event conferring resistance to oxidative stress [18]. Nevertheless, after IRI, proliferation is needed to regenerate the damaged renal cells. In earlier murine experiments, we showed that fasted mice resume normal food intake immediately after surgery and show significant weight gain [16]. This restoration to normal diet activates mTOR and promotes cell proliferation. mTOR activity in our study remained suppressed up to 48 h after IRI. Perhaps due to this extended mTOR inhibition, damaged cells cannot be regenerated, resulting in irreversible kidney failure. This suggests that the timing of mTOR inhibition determines the difference between attenuating or ameliorating IRI. Another explanation for differences in results in rapamycin studies is that the administration of rapamycin differs. Longevity experiments administered rapamycin in microencapsulated form mixed with food, while others report intraperitoneal injections [19]. Difference in pharmacokinetics and bioavailability, caused by different administration techniques, may induce different effects. We administered a single dose of rapamycin intraperitoneally 24 h before IRI. Both the dosages, as well as the route, timing, and number of administrations, may influence the outcome. In addition to our in vivo study, our in vitro experiments did not show any protective effects of rapamycin against oxidative stress.

Conclusion

mTOR inhibition by rapamycin before the induction of renal IRI does not mimic the protective effect against IRI induced by DR. The difference in kinetics of mTOR inhibition and activation between DR and refeeding and

pharmacological inhibition of mTOR may underlie this difference.

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HK-2 cells were kindly provided by Prof. van Kooten, Leiden University Medical Center, Leiden, The Netherlands. The PTECs were kindly provided by Prof. Baan, Erasmus Medical Center, Rotterdam, The Netherlands. Both cell lines were not a primary cell line.

Statement of Ethics

All the animal experiments were performed after the approval by the University Animal Experiments Committee (Dutch Ethical Committee, Protocol No. 105-11-08) under the Dutch National Experiments on Animals Act, compiled with Directive 86/609/EC (1986) of the Council of Europe. The immortalized cell lines used in this study were provided by Leiden University Medical Center (HK-2) and Erasmus Medical Center (PTEC) (as stated above). Ethical approval for the use of these cells is not required in accordance with local/national guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Eline van den Akker, Ron W.F. de Bruin, Jan N.M. IJzermans, and Frank J.M.F. Dor participated in the design and interpretation of the study and drafting of the manuscript and approved the final version.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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