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The Impact Of Resveratrol On The Interplay Between The Hippo And The microRNA 21/Programmed Cell Death 4 (PDCD-4) Signaling Pathways In Acetaminophen Induced Acute Renal Injury In Rats

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- yes-associated protein 1
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- apoptosis

Abstract

Background: Resveratrol is a polyphenol with antioxidant properties.The study aims to clarify the resveratrolimpact on acetaminophen-induced acute kidney injury in rats through targeting the hippo signaling pathway and the microRNA 21/Programmed cell death 4 (PDCD-4) axis. **Methods:** 24 male albinorats were enrolled into 3 groups, with8 ratsin each group. Control group received normal saline, oncedaily, for 7 days.Acetaminophengroup has been treated with a single dose of acetaminophen (2g/Kg) orally 24 hours before being sacrificed. Resveratrol group waspretreated with resveratrol (30 mg/kg)for 7 days, before being given acetaminophen orallyas single dose. The gene expression levels of PDCD- 4 and microRNA 21,and levels of yes-associated protein 1 (YAP1)were estimated in renal tissue. Immunohistochemical expression of BAX wasdetermined. Blood urea, serum creatinine, malondialdehyde(MDA),and reduced glutathione (GSH)in renal tissue were assayed. **Results:** Acetaminophen group displayed significant rise inthe levels of MDA, YAP1,blood urea, serum creatinine, andBAX.PDCD-4 and microRNA 21 gene expression levels were elevated in acetaminophen group, in comparison with other groups,while resveratrol group showed significant decrease in the same parameters. Acetaminophen group displayed significant decrease in serum BCL2, and tissue GSHcompared to other groups. **Conclusion:** Resveratrol has a renoprotective efficacy through reducing oxidative stress, targeting thehippo signaling pathway effector YAP1, and the microRNA 21/PDCD-4 signaling pathway, decline of BAX which is pro-apoptotic, and elevation of BCL 2 which is anti-apoptotic.

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Introduction

Acetaminophen was considered as one of the most popular antipyretic and analgesic drugs in the world.(1). The cytochrome p-450 enzyme converts 2-4% of therapeutic doses of acetaminophen into the hazardous metabolite N-acetyl p-benzoquinone (NAPQI) (2). Excess NAPQI causes damage to the liver and kidneys (3).

It was demonstrated that N-acetylcysteine can be utilized to treat hepatotoxicity caused by acetaminophen. Although N-acetylcysteine raises the amount of glutathione stored in the liver, it cannot shield the kidneys from the effects of an acetaminophen overdose (4). Furthermore, acetaminophen has been shown to trigger apoptosis by triggering the caspase cascade (5).

Yes-associated protein 1 (YAP1) is a necessarydownstream signaling target of hippo pathway. YAP1 regulates cell proliferation,apoptosis, tumor differentiation,and metastasis(6). Dephosphorylated YAP moves into the nucleus and attaches itself to the transcriptional enhanced associated domain 1-4(TEAD1-4), to trigger gene expression when the hippo pathway is deactivated (7).

There are at least 12 members of the protein family known as programmed cell death (PDCD) (8). The tumor suppressor known as programmed cell death 4 (PDCD-4) prevents tumor invasion, metastasis, and cellular proliferation (9). It has been confirmed that microRNA 21 (miR-21) modulates PCDC-4 expression in many tissues (8). The B cell lymphoma-2 (BCL 2) is an anti-apoptotic protein. Another important regulator of apoptosis is Bcl-2-associated X protein (BAX), sometimes referred to as BCL 2-like protein 4. Because the BAX/BCL 2 cascade is essential for inducing apoptosis, it attracts a lot of interest from researchers working on targeted therapy and structure-based drug design (10).

Natural products are increasingly being used to treat a variety of diseases in several studies (11). Fruits, vegetables, and grains all contain the polyphenolic antioxidant resveratrol (12,13). It has been well documented to have strong cardiovascular preventive and therapeutic benefits through scavenging reactive oxygen species (ROS) (14), anti-inflammatory actions (15), and suppression of lipid peroxidation. The present study aims to clarify the impact of resveratrolon acetaminophen-induced acute kidney injury in rats through targeting the hippo signaling pathway and the microRNA 21/PDCD-4 signaling pathway.

MATERIALS AND METHODS

Drugs and chemicals:

Acetaminophentablets of (Paramol®) (500 mg) from Misr Co., Pharmaceutical Industries, Cairo, Egypt,were disintegrated in saline.Resveratroland utilized chemicals were bought from company of Sigma Chemicals (St. Louis, MO, USA). High grade chemicals were utilized in the study.

Animals and experimental design:

The current study was conducted using 24 male albino rats aged about 8-week-old, whose weight ranges between 150 - 200 g. Rats were kept in wire mesh cages at 24°C ± 2°C, with 12hours light/dark cycles. Rats had access to water andfood. A period of acclimatization of 1 week was allowed for all rats at the beginning of the experiment.

After acclimatization for 7 days, rats were enrolled into three groups of 8 rats each. Normal salineand 1% carboxy methyl cellulosewere administratedby oral gavage to control group, one timeper day, for 7 days. Rats of (acetaminophen group)received only one dose of acetaminophen (2 g/kg) orally,whichwas prepared by suspending in 1% carboxy methyl cellulose solution(16).Rats of (resveratrol/acetaminophen group)were pretreated for 7 days with resveratrol (30 mg/kg) before being given a single dose of acetaminophen orally,this is according to the study design ofDallak M et al (17).Resveratrol was prepared at a concentration of 20 mg/ml using 0.5% carboxymethyl cellulose as the vehicle and administered by oral gavage(18).Then,rats were euthanized 24 hours after drug regimen administration.

Ethical considerations:

The research follows the guidelines of committee of ethics of the Faculty of Medicine, Tanta University, Egypt,with the approval code 36264PR1109/2/25.

Sampling:

Blood was collectedby intracardiac puncture,underanaesthesia with thiopental sodium (30 mg/kg).Serum wasseparated and kept at -80 °C to be used for biochemical assay; as blood was collected in a dry sterile centrifugation tube and allowed to clot at room temperature for half an hour, followed by 15 min centrifugation for 3000 rpm at 4°C (19).

After being dissected, kidneys were washed with cold saline solution.Tissue was kept in 10% formalin, for histopathological study. However, tissue was cut into pieces,measured and mixed in phosphate buffer saline(PBS) (pH 7.4)for homogenization (20).The supernatant was gathered and kept at -80°C. Lowry method was used to calculate the total protein content(21).

Assessment of renal function:

Levels of blood ureaand serum creatininewere measured using commercial kits from Sigma-Aldrich, (Cat# MAK006) and (Cat# MAK080) respectively.

Evaluation of redox status,and assay of apoptosis markers:

Colorimetric assay for GSH and MDA in renal tissue,was conducted using commercial kits provided by Biodiagnostic, Egypt(Cat#GR 25 11),and (Cat# GR 25 29)respectively. Immunoassay of BAX and BCL 2 in serum using enzyme-linked immunosorbent assay (ELISA)kits provided by MyBioSource (Cat# MBS2512405),and (Cat#MBS2515143), respectively, according to the protocol of the manufacturer.

Immunoassay of YAP1:

Immunoassay of YAP1 in renal tissue was conducted using ELISA kit obtained from MyBioSource (Cat# MBS077017)according to manufacturer's protocol.

Quantitative Real-Time PCR (qPCR):

Total RNA was isolated using the Gene JET RNA purification kit (ThermoScientific, #K0731, Waltham, MA, USA) from frozen renal tissue. The RNA concentration and purity were evaluated by assessment ofOD260/280, using NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, NC, USA). Revert Aid H Minus Reverse Transcriptase (ThermoScientific, #EP0451, Waltham, MA, USA) was utilized for converting 5 µg of total RNA into cDNA,which acted as template for

SYBR-green qPCR using Step One Plus real-time PCR system (Applied Biosystem, USA). The used primers were: PDCD-4 F: 5′-TGAGCACGGAGATACGAACGA-3′, R: 5′-GCTAAGGACACTGCCAACACG - 3′(22),and microRNA 21 F:5′-GCCTCGTAGGCATCAACGACTG-3′,R:5′GAGTCCTGCGTGTGGCAGCTCG-3′ (23).Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) F:5′-AAGCTCACTGGCATGGCCTT-3′, R:5′-CGGCATGTCAGATCCACAAC- 3′ (22). U6: F:5'-GCTTCGGCAGCACATATACTAAAAT-3',R:5'-CGCTTCAGAATTTGCGTGTCAT-3' (21).GAPDH and U6 act as housekeeping genes. The relative gene expression was conducted using $2^{-\Delta\Delta Ct}$technique (24).

Histopathological Evaluation:

- Light microscopic study:

Tissue preparation began with a 24-hour fixation in 10% neutral buffered formalin (25), followed by tissue dehydration, xylene clearing, and paraffin embedding to enable specimen storage in a wax block that can be sectioned on a microtome, which creates extremely fine sections that are then placed on microscope slides.

- Hematoxylin and Eosin staining (H&E staining):

Normal histological structure and the pathological changes of the rat's kidney specimens among experimental groups were examined usinghematoxylin and eosin stain,according toBancroft J.D. and Gamble M(26).

First, xylene was used for dewaxing the slide. Then rehydration by immersing the slides in decreasing alcohol concentrations, ranging from 90%, 70% to50%. After that, distilled water was added to the slides. Sections were then stained for 15 minutes in hematoxylin and rinsed for 10 minutes in tape water. Then,they spent a minute being stained in eosin This results in staining the nuclei with blue color,while the cytoplasm appeared with variable degree of pink.

- Bax immunohistochemistry:

Deparaffinization of sections in xylene and rehydration in graded ethanol were followed by pre-treatment with 10 mM citric acid buffer (pH = 6) in a microwave (Eletrolux, SP, Brazil) for 3 cycles (5 min each) at 850 W for antigen retrieval. After that, they were pre-incubated with 0.3% hydrogen peroxide in PBS for 5 min,and then tissues were blocked with 5% normal goat serum in PBS for 10 min. The specimens were then incubated with anti-BAX polyclonal antibody overnight within the fridge at 4 °C at a concentration of 1:200. Incubation was followed by 2 washes in PBS for 10 min. Next, Sections were treated for one hour at a dose of 1:200 in PBS with a biotin-conjugated secondary antibody against rabbit IgG. Sections were then subjected to two PBS washes before being treated for forty-five minutes with Vector Laboratories' premade avidin biotin complex coupled to peroxidase. A 0.05% solution of 3-3′-diaminobenzidine solution was applied to see the bound complexes, and Mayer'shematoxylin was used as a counterstain for two to five minutes. (27,28).

Statistical analysis:

The Statistical Program of Social Sciences (SPSS) for Windows, version 20 (Armonk, NY: IBM Corp.), was utilized.The Shapiro-Wilk test

was used to detect ifthe data were normally distributed or not (29).The values of measured parameters were expressed asmean ± SD . The three groups under study were compared using the one-way ANOVA test, and pairwise comparisons were made using the Post Hoc test. At $P < 0.05$, the difference was significant.

Results:

Resveratrol effect on renal function:

Acetaminophen administration induced significantrise in the levels of blood urea and serum creatinine in comparison with the control group. Resveratrol treatment significantly decreased their levels as compared to the acetaminophen group,as illustrated in table 1.

Resveratrol impact on redox status, YAP1 and apoptosis markers:

As regards to oxidative stress parameters administration of acetaminophen induced a significant decline in tissue GSH and a significant elevation in tissue MDA as compared to control group. Resveratrol treatment induced significant increase in tissue GSH and significant decline of MDA levels,when compared with the acetaminophen group,as illustrated in table 1.

The current study displayed significant rise in the levels of YAP1 and BAX proteins and a significant decrease of BCL2 protein after administration of acetaminophen as compared to the control group.Treatment withresveratrol significantly decreased tissue levels of YAP1 and BAX proteins and significantly increased BCL2 protein level as compared to the acetaminophen group,as illustrated in table 1.

Effect of resveratrol on gene expression of miR- 21 and PDCD-4:

Acetaminophen administration induced a significant rise in miR-21 gene expression as compared with the control group while PDCD-4 gene expression showed the reverse. Resveratrol treatment significantly decreased miR-21 gene expression and significantly increased PDCD-4 gene expression as compared to the acetaminophen group,as illustrated in table 1.

Table (1): Comparison between the three studiedgroups according to different parameters.

	Control	Acetaminophen	Resveratrol + acetaminophen	p
Urea (g/dl)	21.95 ± 1.50	53.58[a] ± 1.19	31.51[ab] ± 0.96	<0.001[*]
Creatinine (mg/dl)	0.34 ± 0.03	0.82[a] ± 0.03	0.54[ab] ± 0.03	<0.001[*]
GSH (nmol/gm protein)	38.55 ± 1.03	19.25[a] ± 0.64	33.75[ab] ± 1.48	<0.001[*]
BCL2 (ng/ml)	2.90 ± 0.20	1.45[a] ± 0.12	2.39[ab] ± 0.11	<0.001[*]
BAX (ng/ml)	0.50 ± 0.03	1.89[a] ± 0.10	0.77[ab] ± 0.03	<0.001[*]
miR-21	1.08 ± 0.11	2.61[a] ± .18	1.79[ab] ± 0.08	<0.001[*]
YAP1 (pg/mg protein)	52.55 ± 2.37	160.1[a] ± 3.02	82.54[ab] ± 1.32	<0.001[*]
PDCD-4	1.05 ± 0.12	0.49[a] ± 0.08	1.13[ab] ± 0.32	<0.001[*]
MDA (nmol/gm)	7.68 ± 0.32	16.26[a] ± 0.47	9.30[ab] ± 0.55	<0.001[*]

Data was expressed using Mean ± SD.
*: Statistically significant at $p \leq 0.05$
a: Significant with control group
b: Significant with acetaminophen group

Histopathological study:

Hematoxylin and eosin-stained sections of control group demonstrated normal structure of renal corpuscles,showing thin-walled Bowman's capsule with apparent glomeruli and urinary space between them. The proximal convoluted tubules appeared consisting of pyramidal cells and the distal convoluted tubules consisting of cuboidal cells with clear nuclei. The distal convoluted tubules had wider lumens than the proximal convoluted tubules.Acetaminophen group sections showed marked vacuolations with dilated and congested blood vessels and other sections illustrated wider urinary space than control group. The proximal and distal convoluted tubules appeared with atrophic nuclei. Sections from acetaminophen/resveratrol group showed return to normal structure of kidney with apparently normal Bowman's capsules, normal glomeruli and urinary space. However, some proximal and distal convoluted tubules were still showing some necrotic cells,as illustrated in fig 1.

BAX immunohistochemical staining of kidney sections from control group showed negative results. Sections from acetaminophen group demonstrated high expression of BAX immunohistochemical staining which appeared as excessive brown discoloration. In acetaminophen/resveratrol group sections showed less expression of BAX, but it is still higher than control group (brown discoloration was present but less than acetaminophen group, as illustrated in fig 2.

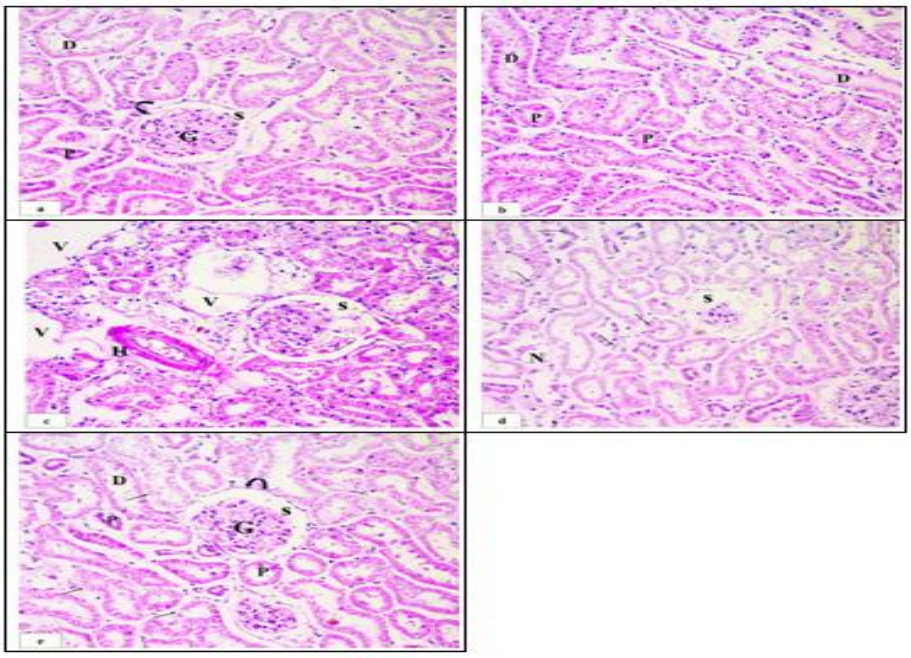

Fig 1: a) photomicrograph of a section of kidney of control group demonstrating renal corpuscles consisting of thin-walled Bowman's capsule (curved arrow), glomeruli (G) with urinary space (S) between them. b) In Control group sections, the proximal (P) convoluted tubules are clearly apparent consisting of pyramidal cells and the distal convoluted tubules (D) consisting of cuboidal cells with clear nuclei. The distal convoluted tubules have wider lumens than the proximal convoluted tubules (H&E, x400). c) Photomicrograph of a section of kidney of acetaminophen group showing marked vacuolations (V) with dilated and congested blood vessels (H). d) Another section from the acetaminophen group illustrates wider urinary space (S) than control group and proximal and distal convoluted tubules with atrophic nuclei (thin arrows) (H&E,x400). e) Sections from resveratrol/acetaminophen group shows apparently normal Bowman's capsules (curved arrow) with normal glomeruli (G) and urinary space (S). However, some proximal (P) and distal convoluted tubules (D) still show some necrotic cells (H&E, x400).

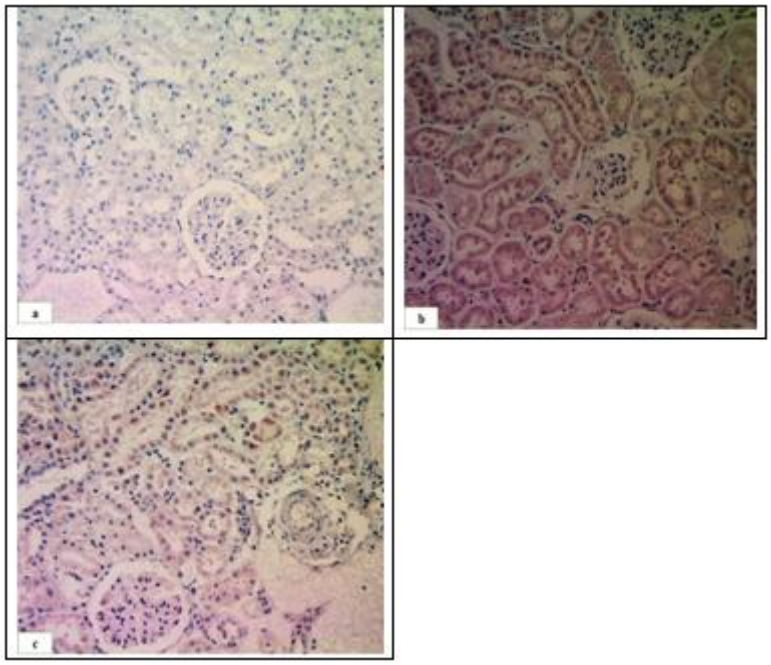

Fig 2: a) BAX Immunohistochemical staining of kidney sections from control group shows negative results. b) Sections from acetaminophen group demonstrates high expression of BAX immunohistochemical staining. c) Resveratrol/acetaminophen group shows less expression of BAX, but it is still higher than control group (BAX immunostaining, x400) .

Discussion:

Acute kidney injury (AKI) is estimated to affect millions annually (30). Resveratrol is a naturally occurring polyphenolic chemical that has anti-oxidant,and anti-inflammatory effects. (31). This work aims to elucidate the effect of resveratrol on acetaminophen induced AKI in rats through targeting YAP1 and the microRNA 21/PDCD-4 signaling pathway.

Acetaminophen is a widely available analgesic and antipyretic drug (32). In the current study, acetaminophen administration at high dose is used to induce acute nephrotoxicity, which was indicated by statistically significant increase of serum creatinine and blood urea levels in acetaminophen group in comparison with the control group,and by the histopathological study.In addition to the redox imbalance caused by acetaminophen administration, as it showed significant elevation ofMadmanreduction in GSH levels compared to resveratrol and control groups. This is in harmony with the study ofEassawy MMT et al (32). A possible explanation could be that, acetaminophen is converted by the microsomal P-450 into the intermediate NAPQI,which is extremely reactive. Cellular GSH is depleted with acetaminophen overdoses,as GSH reacts immediately with NAPQI. This makes it possible for NAPQI to attach to proteins in cells and start lipid peroxidation, which damages the kidneys(33).

Furthermore, oxidative stress is a potent inducer of apoptosis. Overdose of acetaminophen causes renal injury by oxidative stress and induction of apoptosis. Acetaminophen group showed statistically significant increase in BAX protein level and

statistically significant decrease of BCL 2 protein this aligns with Najafizadeh et al. who reported that administration of high dose acetaminophen can trigger the apoptosis cascade by overexpressing pro-apoptotic proteins in the kidney tissue, such as cleaved caspase-3 and BAX proteins, instead of the anti-apoptotic BCL 2 protein(34).

Resveratrol showed renoprotective effect, as resveratrol administration resulted instatistically significant decline inserum creatinine and blood urea levels,in relation to acetaminophen and control groups.Moreover, the histopathological study showed restoring normal renal structure in resveratrol/acetaminophengroup, althoughthe appearance of some necrosis in proximal and distal convoluted tubules. This aligns with the findings of Lan et al who clarified the protective effects of resveratrol in maintaining renal function, alleviating inflammation, decreasing oxidative stress, and preventing cell death in ischemia-reperfusion renal damage(35). According to Alaasam et al, resveratrol administration reduced blood urea and serum creatinine levels, reduced caspase-3, increased GSHand BCL2 (36).

The current study displayed significant increase in reduced glutathione level,and significantdecline in level of MDA,in resveratrol /acetaminophen group,in comparison with the other two groups.This is in harmony with the findings of Chupradit et al., who concluded that resveratrollowers oxidative stress in hepatic tissue by raising GSH levels and drastically lowering MDA, because of its anti-inflammatory and antioxidant qualities (37). Also resveratrol/acetaminophen group showed statistically significant decrease in BAX protein level and statistically significant rise of BCL 2 protein compared to acetaminophen and control groups. In accordance, Yalçin et al., concluded that resveratrol treatment may induce an antiapoptotic effect by regulating the BAX/BCL 2 ratio (38).

Yes-associated protein 1(YAP1) is an important effector target of the hippo signaling pathway, which is implicated in cell proliferation, differentiation, and apoptosis (39).Acetaminophen group showed statistically significant increase of YAP1 protein level compared to resveratrol and control groups. In accordance Xu et al., found that YAP1 protein levels risethrough the stage of AKI repair (40). While prolonged YAP activation in severe AKI may result in maladaptive repair and the development of chronic kidney disease, transient activation of YAP during the acute phase of AKI may promote renal recovery and regeneration.(41). YAP1 can trigger apoptosis through binding p73 instead of transcriptional enhanced associated domain TEAD and leads to upregulating the pro-apoptotic gene BAX (42). We found that there was statistically significant decreased of YAP1 level by resveratrol treatment compared to acetaminophen and control groups. The findings presented in the current study align with the research of Qin et al (43).

MicroRNA-21 affects cell cycle and apoptosis. Acetaminophen group showed statistically significant rise of miR-21 gene expression when compared to the other 2 groups. The findings presented here align with the research of Sun et al, who declared that miR-21

was significantly upregulated in activated fibroblasts,and that overexpression of miR-21 promotes the progression of renal fibrosis(44). According to Sun Pengfei et al miR-21 triggers transforming growth factor-β (TGF-β) signaling which induces fibrosis (45).

In the present study There was a statistically significant decrease of miR-21 relative gene expression in resveratrol/acetaminophen group, compared to the two other groups. This could be supported by the study of ShamsEldeen et al., who concluded that the bile duct ligation group demonstrated an increased in the expression of miR-21 in the liver that was significantly inhibited by resveratrol(46). Huffman et al suggestedthat a new strategy to lessen acataminophen-induced hepatotoxicity and improve survival during the regeneration phase could be throughmiR-21 suppression(47). According to Sun et al.,miR-21 enhances renal fibrosis.Therefore, targeting miR-21 attenuates fibrotic lesion progression (44).It is noteworthy that, acute renal injury may progress into interstitial renal fibrosis and chronic kidney disease (48).

Programmed cell death protein 4 (PDCD-4) regulates several essential processes ranging from embryonic development to normal tissue turnover. The current study displayed significant decrease of PDCD-4 gene expression level in acetaminophen group. This aligns with the research of Sun et al, which proposes that miR-21 maintained constant levels by employing an auto-regulatory loop between miR-21, PDCD-4 and activator protein-1(AP-1)(44). According to Sun Pengfei et al, miR-21 has direct inhibitory effect on PDCD-4 (45).

The present research depicted that resveratrol resulted in significant increase in PDCD-4 relative gene expression in comparison with the other two groups. This could be supported by the research of Keshavarz Motamed et al., who declares that resveratrol causes downregulation of miR-21accompanied by upregulation of PDCD-4 in cancer (49).

On the contrary, Ma et al., suggested that PDCD-4 may cause radiotherapy-induced AKI in rectal cancer by triggering apoptosis, activating the nuclear factor kappa B signaling pathway, and promoting an oxidative stress response. However, the research of Ma et al neglected the study of additional molecular targets that could improve the understanding ofthe complex mechanisms included in AKI (50).

Conclusion:

Collectively, the current research implied that resveratrol has a renoprotective efficacy through reducing oxidative stress, targeting pro-apoptotic BAX, anti-apoptotic BCL2, and the key effector of the hippo signaling pathway YAP1,inaddition to targeting the microRNA21/PDCD-4 signaling pathway in acute kidney injury induced by acetaminophen in rats.

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