Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2022.906

SIRT1720 promotes survival of corneal epithelial cells via the P53 pathway

Hayder AbdulHasan Jalil[1], Basma Talib Al-Sudani*[2], Ghaith Ali Jasim[1]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Iraq

[2]Department of Clinical Laboratories Sciences, College of Pharmacy, Mustansiriyah University, Iraq

***Corresponding author:** Basma Talib Al-Sudani, Department of Clinical Laboratories Sciences, College of Pharmacy, Mustansiriyah University, Iraq. Email: basma_alsudani@uomustansiriyah.edu.iq

Submitted: 23 March 2022; Accepted: 18 June 2022; Published: 16 July 2022

ABSTRACT

Purpose: To investigate the protective role of SRT1720 (SIRT1 activator) against the oxidative stress caused by H_2O_2 in the corneal cell line.

Methods: Human corneal (2.040 pRSV-T) cell lines were cultured and treated with SRT1720 (as SIRT1 activator) and nicotinamide (NAM, a SIRT1 inhibitor), and incubated with H_2O_2. The expression level of SIRT1, p53, and acetyl-p53 was measured by western blot. Propidium iodine/annexin V-FITC staining, and flow cytometry was used to evaluate apoptosis. The trypan blue assay was used to assess the morphological modifications that occurred after the treatment, and Pifithrin-α (PFT-α) was used to inhibit the p53 pathway.

Results: The investigation revealed that under oxidative stress, SRT1720 caused a reduction in acetyl-p53 expression and increased SIRT1 expression. It was also found that under oxidative stress, SRT1720 suppressed apoptosis. In comparison, NAM promoted cell apoptosis under oxidative stress. NAM's destructive effect was eliminated by PFT-α, a suppressor of the p53 pathway. PFT-α reduced the morphological changes in 2.040 pRSV-T cell lines compared to NAM treatment and inhibited apoptosis.

Conclusions: The protective effects of the SIRT1 activator (SRT1720) indicate that H_2O_2 induces oxidative stress-associated cell damage. The results also encouraged us to consider using SRT1720 to improve corneal safety and reduce the adverse effects of oxidative damage.

Keywords: *2.040 pRSV-T cell line, Acetyl-p53, SIRT1, SRT1720*

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

INTRODUCTION

The cornea is the outermost part of the eye that serves to protect the eye from mechanical injury.[1] The cornea receives a considerable quantity of additional environmental oxygen and daylight, especially the UV spectrum, due to its highly exposed location.[2] These factors cause the generation of reactive oxygen species (ROS), and as a result, oxidative stress in the cornea.[3] Any defect in the equilibrium between the generation of ROS and the detoxification ability of corneal cells causes oxidative stress.[4] Although medicines with antioxidant characteristics such as vitamin E and C are widely used to treat corneal damage caused by oxidative stress agents,[5] the increasing evidence to catalyzing the development of novel therapeutic agents, where the targeting inhibiting p53-dependent apoptosis to treat corneal disease. Finding methods for preservation of corneal damage against apoptosis caused by oxidative stress is consequently therapeutically important in the prevention of eye diseases. Numerous acetylation sites on the p53 protein stabilize and activate it, allowing it to promote apoptosis.[6] SIRT1 deacetylation of p53, on the other hand, prevents apoptosis caused by oxidative damage and DNA impairment.[7] SRT1720 is a small molecule activator SIRT1, that is structurally distinct from resveratrol. A series of experimental SRT drugs (SRT1720) were reported to activate SIRT1.[8] These small molecules are a potent activator of SIRT1 which is 1000 times more potent than resveratrol.[9] Previous studies have provided evidence for the potential clinical use of SIRT1-activating compounds such as resveratrol to protect corneal epithelial cells from the adverse effects of ROS.[10] But, the mechanisms of resveratrol against cornea protection by the activation of SIRT1 were unclear because the resveratrol characteristics at several levels, including cellular and molecular signaling, and physiological impacts were toxic and unstable. The SIRT1 protection mechanism includes the deacetylation of targets such as p53, the forkhead box class O (FoxO) family, peroxisome proliferator activated receptor γ, coactivator 1α (PGC-1α), and nuclear factor Kappa B(NF-κB).[11] Suppressing p53-dependent apoptosis is a significant downstream mechanism among these targets.[11] Several acetylation sites are involved in p53 activation and stabilization to induce apoptosis.[12,13] SIRT1 deacetylation of p53, on the other hand, protects cells from apoptosis in relation to DNA degradation and oxidative damage.[14,15] The aim of this study was to investigate whether the SIRT1 activator (SRT1720) will protect cornea from oxidative stress via the inhibition of the p53 pathway. The results revealed that the etiology of SIRT1 protection involves the inhibition of the p53 pathway, and SRT1720 can be used in the future to prevent oxidative damage to cornea.

EXPERIMENT

Materials

The chemicals required for the experiment were purchased from ThermoFisher Scientific and Sigma-Aldrich. Human corneal epithelial cell line (2.040 pRSV-T) was purchased from the American Type Culture Collection (ATCC). Keratinocyte-serum free medium (17005-042) was purchased from Gibco-BRL, and BCA protein conc. Kit was purchased from the Elabscience, USA. Anti-p53 (ab131442), anti-p53 (acetyl K370) (ab183544), and anti-SIRT1 antibody (ab7343) were purchased from Abcam. Goat anti-mouse IgG secondary antibody was purchased from Invitrogen. Western blot and SDS kits were purchased from Elabscience, and Annexin V Apoptosis Detection Kit was purchased from Biosciences.

Procedures

Cell lines and cultures

2.040 pRSV-T cells were cultured in 75 cm^2 flasks with keratinocyte-serum free medium supplemented with 5 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, 0.005 mg/ml

insulin, and 500 ng/ml hydrocortisone; 0.25% trypsin/EDTA in HBSS, without Ca^{2+}/Mg^{2+} and incubated in 5% CO_2/ 95% humidified air at 37°C.[16,17]

Oxidative stress model and treatment of the activator and inhibitor of SIRT1

Human 2.040 pRSV-T cells were split into five main groups: (1) the control group, where 2.040 pRSV-T cells were cultured in normal conditions; (2) the H_2O_2, where 2.040 pRSV-T cells were treated with H_2O_2 at an ultimate concentration of 400 µmol/L; (3) the SIRT1 activator SRT1720 group, which includes four different sub-groups, where 2.040 pRSV-T cells were pre-treated with SRT1720 at final concentrations of 1, 3, 5, 7, 9, 11, and 13 µM, for 12 h, before adding 400 µmol/L H_2O_2; (4) the SIRT1 inhibitor Nicotinamide (NAM) group (NAM group), in which 2.040 pRSV-T cells were pre-treated with NAM at final concentrations of 100µM, 12 h prior to adding H_2O_2 to an ultimate concentration of 400 µmol/L. These groups were equilibrated for 24 h.

Western blot analysis

The proteins were separated via SDS acrylamide gel electrophoresis. The specific proteins (p53, Acetyl-p53 and SIRT1) were identified from a complex mixture of proteins extracted from cells by western blot technique.[18]

Sample preparation

1. Protein extraction: In 6-well plates, the normal corneal epithelial 2.040 pRSV-T cell lines were planted at the rate of 20,000 cells per well. The medium was removed by washing the normal corneal 2.040 pRSV-T cells three times with pre-cooled PBS (0.01 M, pH 7.4). After that, the human corneal epithelial 2.040 pRSV-T cell lines were lysed on ice for 1 h by adding 1mL from RIPA lysis buffer (a traditional rapid cell lysate used as the preferred lysate for protein extraction from cells in the western blot assay that continues: 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% C24H39O4Na, 1 mM EDTA, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, 1 mM PMSF). All cell samples were sonicated for 1 min under an ice water bath with 2 s' sonication and 2 s' intervals to completely lyse the cells and the viscosity of the sample, then centrifuged at 12,000 rpm for 10 min at 4°C. In the end, supernatant was taken and measured for protein concentration by BCA Protein Colorimetric Assay Kit.
2. Protein concentration measurement: To measure the total protein, a BCA protein concentration kit was used. Firstly, a serial concentration was prepared to get a standard curve dilute by adding 0, 0.2, 0.3, 0.4, 0.6, 0.7, 0.9, 1 mg/mL from BCA standard solution with normal saline. In 96-well plate, 20 µL of standard solution at different concentrations was added. Secondly, 18 µL PBS and 2 µL samples supernatant were added to 200 µL of BCA working solution to the wells. It was mixed for 20 seconds and incubated at 37°C for 30 min. Finally, the OD value of each well at 562 nm was measured with a microplate reader. Then, the protein concentration in each samples were calculated according to the formula:

$$\text{Protein content (mg/mL)} = (\Delta A562 - b) \div a \times f$$

(Where, a: The slope of standard curve; b: The intercept of standard curve; f: Dilution factor of sample before test).

The total protein amount of samples was between 70-85µg.
3. Sample denaturation: Protein samples supernatant were mixed with 5× SDS buffer according to the ratio 4:1 (4µl samples + 1µl 5× SDS buffer) and incubated in heating block at 95◦C for 10 min. for the prepared of marker: 5 µL volume of pre-stained

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

protein marker was used. After that, the samples were added to the microgel (1 mm thick mini-gel).

Western blot experiment procedure

1. Gel preparation: The molecular weight of p53 protein and SIRT1 are 53 and 110 KDa respectively. Therefore, 6% SDS gel electrophoresis was prepared. Briefly, the separating gel was prepared by added 5.3 ml ddH_2O, 2 ml 30% Acr-Bis (29:1) and 2.6 ml separating gel mix in a clean beaker and mixed. To polymerize the gel, 0.1 ml from 10% APS and 0.008 TEMED were added to the first mixture and mixed gently to avoid bubbles. After that, the mixture gel was added to the assembled gel mold immediately. Then, 1ml absolute alcohol was added on the separating gel to flatten the separating gel level, and kept at RT for 30-60 min until the separating gel is solidified completely. then prepared stacking gel done by added 5.5ml deionized water, 1.3ml 30% Acr-Bis (29:1), 1.08 ml from stacking gel and mixed. 0.08 ml 10% APS and 0.008ml TEMED were added to the stacking solution and mixed gently. The mixture gel is then added to the separating gel immediately. After that, the comb teeth is inserted carefully, and kept at RT for 30-40 min untill it polymerizes completely. Then, the comb teeth is taken out carefully to follow the SDS-PAGE experiment.
2. Adding samples: Tris-Glycine SDS electrophoresis buffer was used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein denaturation. Thus, 250 ml from 1X electrophoretic buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH8.3) was added to electrophoresis tank. The buffer solution must be above sample wells, and the bottom of the gel must be immersed by the buffer solution. 10 µl of samples and pre-stained protein markers were loaded into the gel.
3. Electrophoresis: After loading the samples to the gel, the lid of the electrophoresis tank was covered, minding the positive and negative. The gel electrophoresis was run at voltage 110V for around 2-3 h until bromophenol blue reached the bottom of the gel.
4. Wet transfer: The gel is taken out after electrophoresis and rinsed in cold electrophoretic buffer solution for a few seconds. According to the sandwich mode, the electrical transfer folder were opened, and a sponge mat soaked by transferring the buffers at both sides were stacked up. Then, the qualitative filter paper soaked by transferring the buffers at both sides was putting. the gel flat on the negative filter paper, and then placed flat the PVDF membrane (the PVDF is soaked in methyl alcohol for 5 minutes, then saturated in transferring buffer) on the gel. Then, the transferring buffer was loaded in to the transfer tank, and the folder was inserted. The tank was then kept in ice water. The PVDF membrane was positive pole, and amino acid and protein which were electronegative were moved to the positive pole. The electric voltage of the tank was 200mA. After the transfer, the transferred PVDF membrane was rinsed with a washing buffer at room temperature.
5. Blocking: The rinsed membrane was put into the blocking buffer 5% (w/v) skim milk powder (1mg skim milk powder + 9ml 1X TBST working buffer), and incubated on the shaker at room temperature for 2 h. The TBST working buffer was prepared by mixing these compounds: 145.4 mM NaCl, 10 mM Tirs-base, 0.1% (v/v) Tween 20, pH7.5.
6. Incubation of antibody: After blocking, the membranes were incubated overnight

at 4°C with diluted primary antibody 1:100 (anti-p53, anti-p53 acetyl K379, anti-SIRT1). The membranes were then washed with washing buffer for 10 minutes, and it was repeated thrice. After washing, it was incubated with diluted secondary antibody 1:5000 (anti-mouse IgG) for 2 h, and then washed again for 10 minutes thrice. An improved chemiluminescence method was used to detect the antigen–antibody complexes.

7. Detection: The detection method depends on the label of the secondary antibody. The ECL luminescence detection solution detects bio-macromolecules such as proteins by chemical fluorescence luminescence. The principle is that the horseradish peroxidase (HRP)-labeled antibody directly or indirectly binds to the target protein on the membrane. After washing the membrane, the ECL reagent is added for exposure, and the protein strip can clearly record the luminescence with machine. The working solution was prepared by mixing equal parts of the substrate A and substrate B (0.125 ml from working solution was used per cm^2 of membrane). The blot was incubated in a working solution for 5 min, and then removed from working solution and placed in a plastic membrane protector. The result was then recorded with chemiluminiscence imaging system.
8. Data analysis: The bands were analyzed with Bandscan software.

Measurement of apoptosis by flow cytometry assay

The fluorescein isothiocyanate (FITC) Annexin V apoptosis detection Kit was used to provide a quantitative evaluation of apoptosis. In brief, 2.040 pRSV-T cell lines (1.5×10^5 cells) were grown overnight in 25 cm^2 cell culture flasks before being treated with the SIRT1 activator group 3. SRT1720 was administered to 2.040 pRSV-T cells at ultimate concentrations of 5, 7, 9, and 11μM for 12 h prior to being subjected to 400 μmol/L H_2O_2 for 24, 48, and 72 hours. The positive control has been produced via cultivating control cells for 30 min in a media involving 400 μmol/L H_2O_2. The 2.040 pRSV-T cells were collected, washed repeatedly twice in cool PBS, and reconstituted in a binding buffer (1×10^4 cells/mL). 100 mL of 2.040 pRSV-T cells were incubated for 15 min at 4 °C in the dark with 5 mL of FITC conjugated Annexin V (Annexin V-FITC) and 5 mL of propidium iodide (PI). The stained cells were diluted in a binding buffer and examined on the flow cytometer directly. In each data file, data from 10,000 cells was taken. There are four distinct cell groups that may be clearly differentiated: unmarked (live cells), cells which possess only bound Annexin V FITC (initial apoptotic), cells that have been marked with propidium iodide PI (necrotic), and cells that have both bound Annexin V-FITC and been marked with PI (delayed apoptotic/necrotic cells). The percentage of fluorescent cells in each quadrant was estimated using a two-color dot plot analysis of the fluorescence distribution.[19]

Pifithrin-α (PFT-α) inhibits the p53 pathway

Pifithrin-α (PFT-α) has been used to suppress the p53 pathway (specifically block transcriptional activity of the tumor suppressor p53). Human corneal epithelial 2.040 pRSV-T cell lines were cultured in 6-well plate at the rate of 50000 in each well. After 24 h the cells were treated as four major groups:

1. control group: The 2.040 pRSV-T cell line which was cultivated under normal circumstances.
2. H_2O_2 group: H_2O_2 was used to treat the 2.040 pRSV-T cell line at an ultimate concentration of 400 mol/L.
3. Nicotinamide (NAM) group: 12 hours prior administering H_2O_2 at an ultimate concentration of 400 μmol/L to normal corneal epithelial 2.040 pRSV-T cell lines,

they were pretreated with NAM to an ultimate dose of 100 μM.

4. PFT-α group: PFT-α at ultimate doses of 2.5, 5, and 10 μM, as well as NAM at an ultimate dose of 100 μM, were used to pretreat 2.040 pRSV-T cell lines, 12 h prior to administering H_2O_2 at an ultimate concentration of 400 μmol/L.

The morphology and cell density of corneal epithelial cells after treatment with the p53 pathway inhibitor (PFT-α)

Image-Pro plus software by an inverted microscope was used to determine the 2.040 pRSV-T cells density in each group. 100μl of Trypan Blue was added to 100μl 2.040 pRSV-T cells suspension and mixed well. After 2 min, 1μl of each sample was added to hemocytometer to be analyzed under the microscope. For cell density measurement, one image of each group with the most densely organized cells was selected.[20]

Effects of the p53 pathway inhibitor (PFT-α) on the percentage of apoptotic corneal epithelial cells with NAM and H_2O_2 treatments

In a 6-well plate (1.5×105 cells), human corneal epithelial 2.040 pRSV-T cell lines were planted. Next day, the corneal epithelial 2.040 pRSV-T cells were treated with Pifithrin-α at ultimate doses of 2.5, 5, and 10 μM with 100 μM NAM, 12 h before adding 400 μmol/ L H_2O_2 for 72 hours. The positive control was produced via the cultivation of the control cells in media comprising 400 μmol/L H_2O_2 for 30 minutes. 2.040 pRSV-T cells were collected, washed repeatedly twice with cool PBS, and re-suspended in binding buffer (1×10^4 cells/mL). 5 mL of FITC conjugated Annexin V (Annexin V-FITC) and 5 mL of propidium iodide (PI) were added to 100 mL of 2.040 pRSV-T cells and incubated for 15 min at 4 °C in the darkness. The stained cells were diluted with binding buffer and evaluated on the flow cytometer directly.[19]

Statistical analysis.

All statistical analysis of data was performed using Prism 8 software. A comparison between all groups within the same plate of apoptosis assay were evaluated by one-way ANOVA with Tukey (Prism 8 software). Statistically significant values were considered $p < 0.05$.

RESULTS

Protective effect of SIRT1 against oxidative stress occurs via the p53 pathway by Western Blot

Western Blot analysis of SIRT1 expression (Figure 1) indicates that SRT1720 produced an elevation in SIRT1 expression at different concentrations in H_2O_2-treated cultures. After treatment with SRT1720 at concentrations of 7, 9, and 13 μM, the level of expressions relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) significantly increased. (Figure 1A), in comparison with the H_2O_2 group ($p< 0.0005$) (Figure 2C). In 2.040 pRSV-T cells treated with 1 and 3μM, no such elevation was detected ($p > 0.05$).

Figure 2 shows that in all H_2O_2 groups, the expressions of P53 significantly increased in comparison to the control group ($p< 0.0001$). The expression of Acetyl-p53 was reduced as SRT1720 concentrations increased, as shown in figure 3 ($p < 0.005$), suggesting that acetyl-p53 levels reacted effectively to the upregulation of the expression and function of SIRT1 under oxidative stress.

Measurement of apoptosis by flow cytometry assay

The percentage of apoptotic 2.040 pRSV-T cells significantly increased in the H_2O_2 group to 51.4 ± 1.8 at early apoptotic, and to 22.7 ± 1.7 at late apoptotic ($p < 0.001$), compared with the control group (1.6 ± 0.4) (Figure 4). SRT1720 concentrations up to 7μM significantly decreased the percentage of apoptotic 2.040 pRSV-T cells between 10-4% ($p< 0.005$) in comparison with the H_2O_2 group. But there was

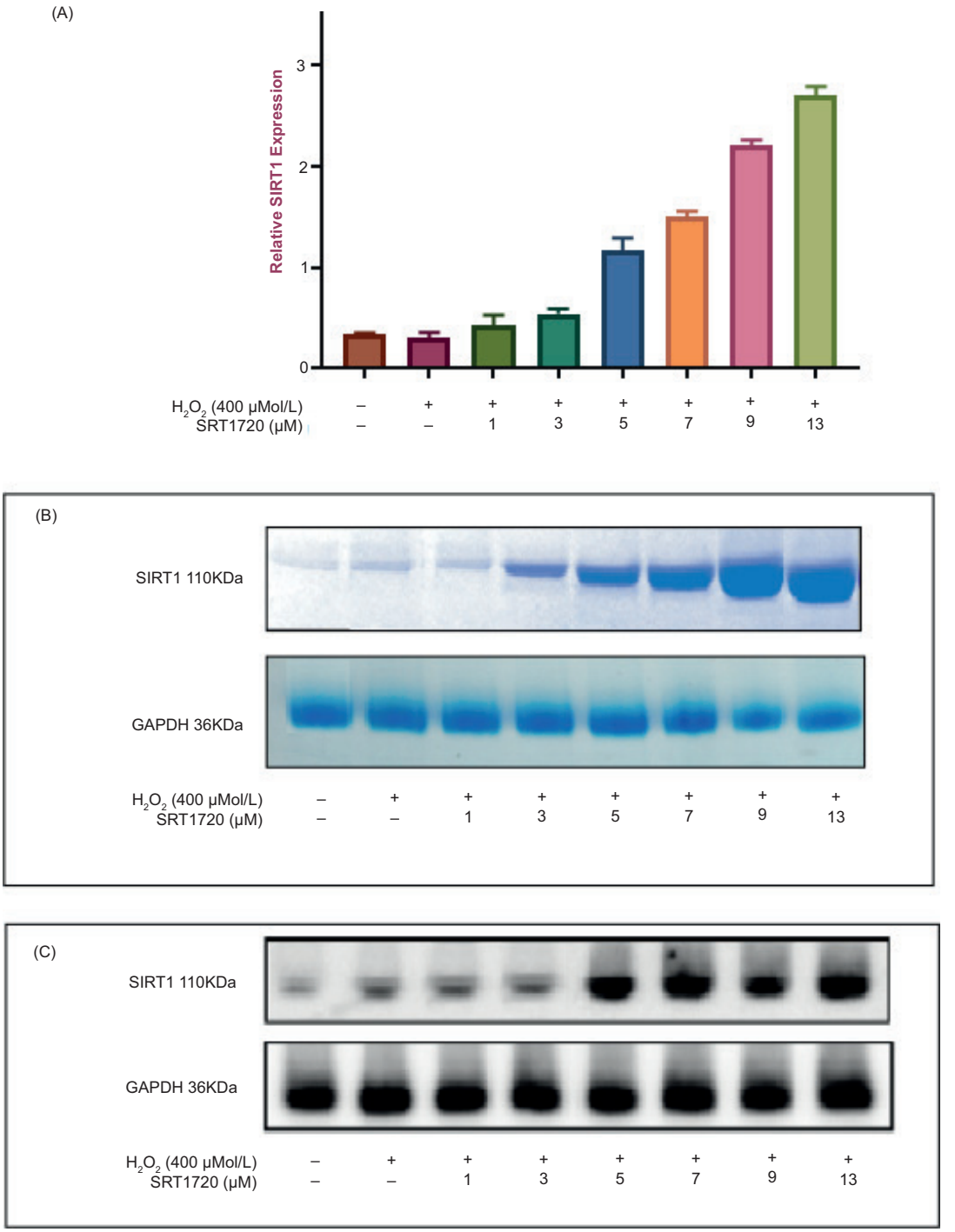

FIGURE 1. Effect of SRT1720 on SIRT1 by Western blot. (A) SRT1720 stimulated a dose-dependent elevation in SIRT1 in H_2O_2-treated cultures. There are labeled concentrations of SRT1720 at the bottom (μM), $p< 0.0005$. (B) SDS PAGE demonstrates the SIRT1 protein separated in 8 samples. (C) Western blot analysis result of SIRT1 expression. Significant difference in comparison to the H_2O_2 group. Y-axis: relative SIRT1 expression versus GAPDH.

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

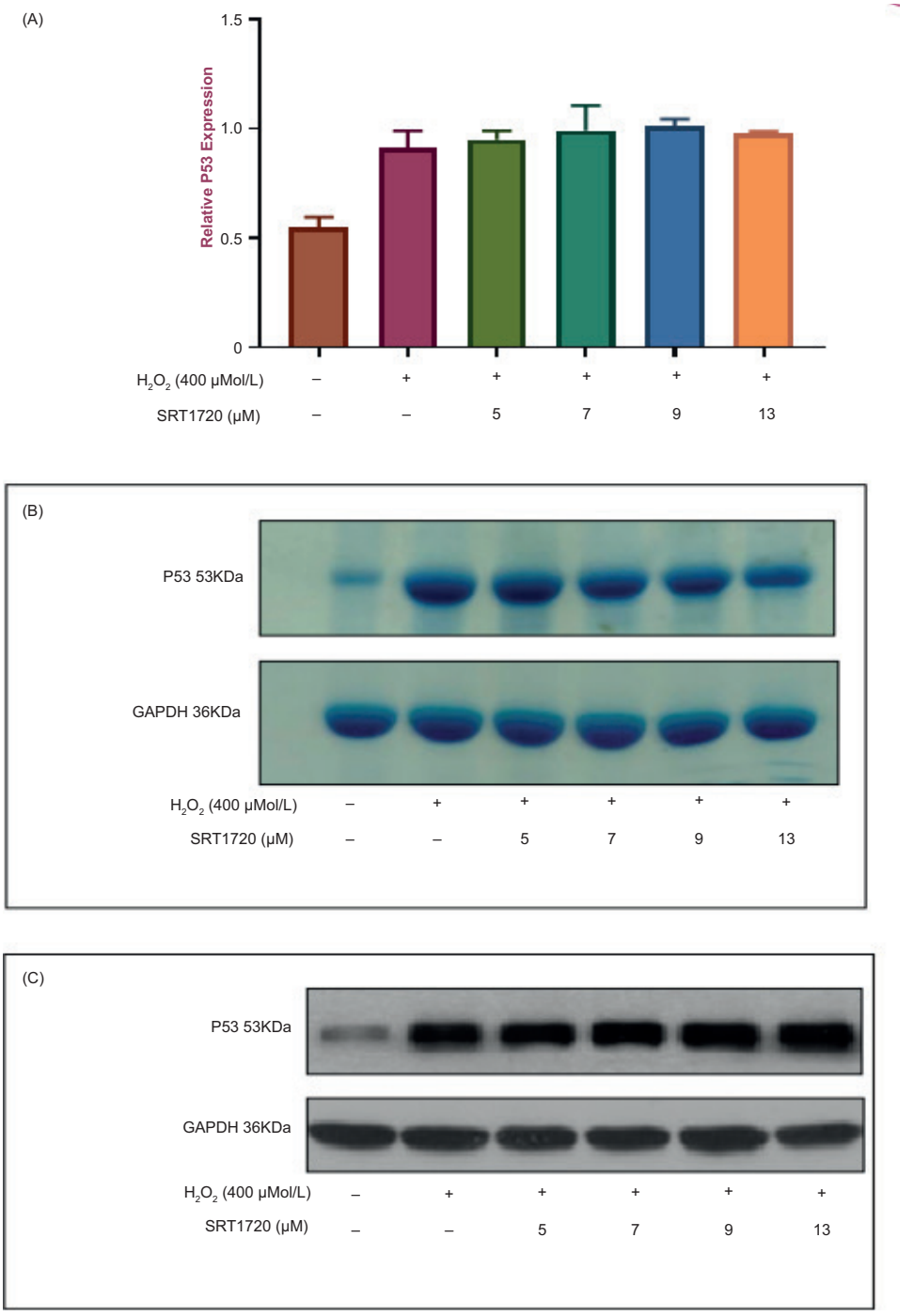

FIGURE 2. Effect of SRT1720 on p53 by western blot. (A) SRT1720 demonstrates increased p53 in all groups under oxidative stress. There are labeled concentrations of SRT1720 at the bottom (µM), $p < 0.0001$. (B) SDS PAGE demonstrates the p53 protein separated in 6 samples. (C) Western blot analysis result of p53 expression reveals that p53 is elevated in all groups under oxidative stress. Significant difference in comparison to the H_2O_2 group. Y-axis: relative SIRT1 expression versus GAPDH.

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

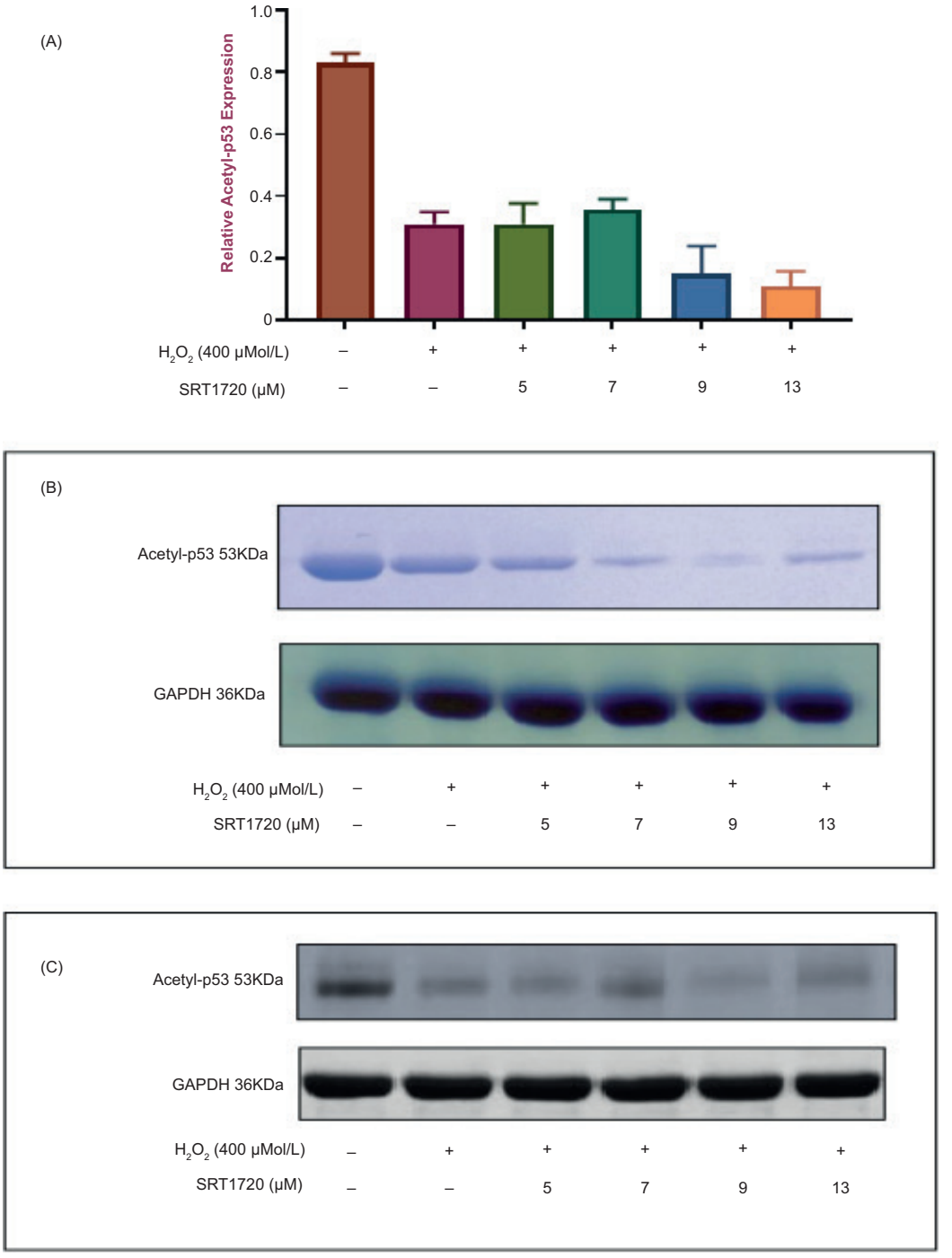

FIGURE 3. Effect of SRT1720 on acetyl p53 by Western blot. (A) SRT1720 demonstrates acetyl p53 decreased by SRT1720 in all groups under oxidative stress. SRT1720 concentrations are labeled at the bottom (µM), $p< 0.005$. B- SDS PAGE demonstrates the acetyl p53 separated in 6 samples. (C) Western blot analysis result of acetyl p53 expression shows that acetyl p53 decreased by SRT1720 under oxidative stress. Significant difference in comparison to the H_2O_2 group. Y-axis: relative SIRT1 expression versus GAPDH.

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

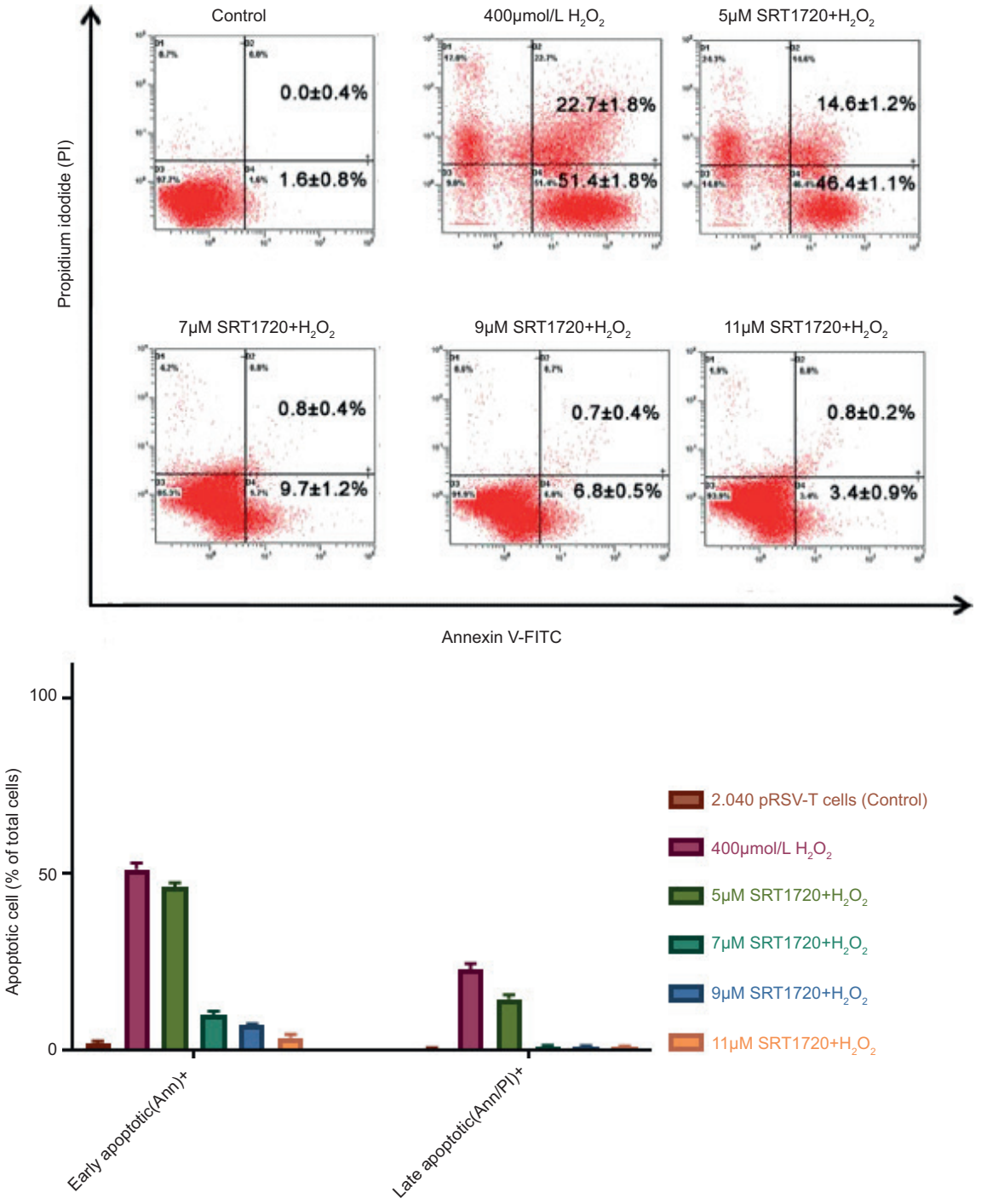

FIGURE 4. Effect of SRT1720 on the percentage of apoptotic 2.040 pRSV-T cell lines. SIRT1720 and H_2O_2 concentrations are labeled at the left (μM and μmol/L). *Significant difference in comparison to the H_2O_2 group, $p< 0.005$ (without SIRT1720).

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

no significant difference between 7, 9, and 11 µM SRT1720 groups with control group ($p > 0.05$).

Inhibition of the p53 pathway by Pifithrin-α (PFT-α)

PFT-α was used to block transcriptional activity of p53 pathway, and NAM was used to inhibit the level of SIRT1 in 2.040 pRSV-T cell lines. Human corneal epithelial cell density was reduced after the treatment with 100M NAM+H_2O_2 (Figure 5), with significant changes in the shape of cells compared to the control group, $p< 0.005$. Nevertheless, when 2.040 pRSV-T cell lines were treated with 5 and 10µM PFT-α in addition to NAM and H_2O_2, the cell density was restored (Figure 5; $p< 0.05$) and displayed the shaped cells, in comparison to the NAM group.

Effects of (PFT-α), a p53 pathway inhibitor on the percentage of apoptotic corneal epithelial cells with NAM and H_2O_2 treatments

To investigate the effects of the p53 pathway inhibitor on the percentage of 2.040 pRSV-T cells, apoptotic, fluorescein isothiocyanate (FITC) Annexin V apoptosis by flow cytometry was used. Figure 6 demonstrates the significantly increased percentage of apoptotic after treating the 2.040 pRSV-T cells with H_2O_2 by 30%, and it increased even more by 20% with the last raise after treating the cells with NAM and H_2O_2, in comparison to

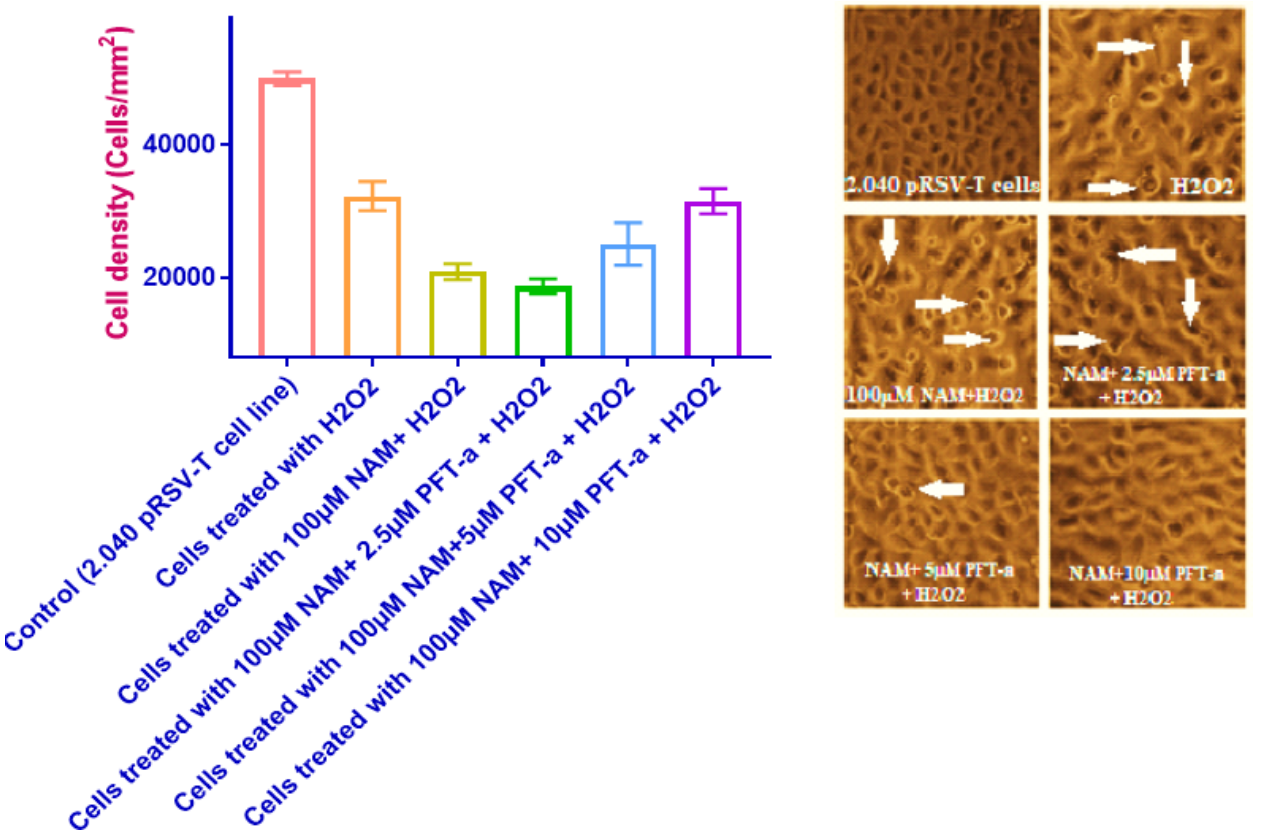

FIGURE 5. Effects of pifithrin-α (PFT-α), a p53 pathway inhibitor, on morphology and cell density in 2.040 pRSV-T cell lines treated with nicotinamide (NAM) and (H_2O_2). Arrow tended to round up and detach from the culture wells and changes in shaped cells. Significant difference in comparison to the H_2O_2+NAM group; $p < 0.05$.

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

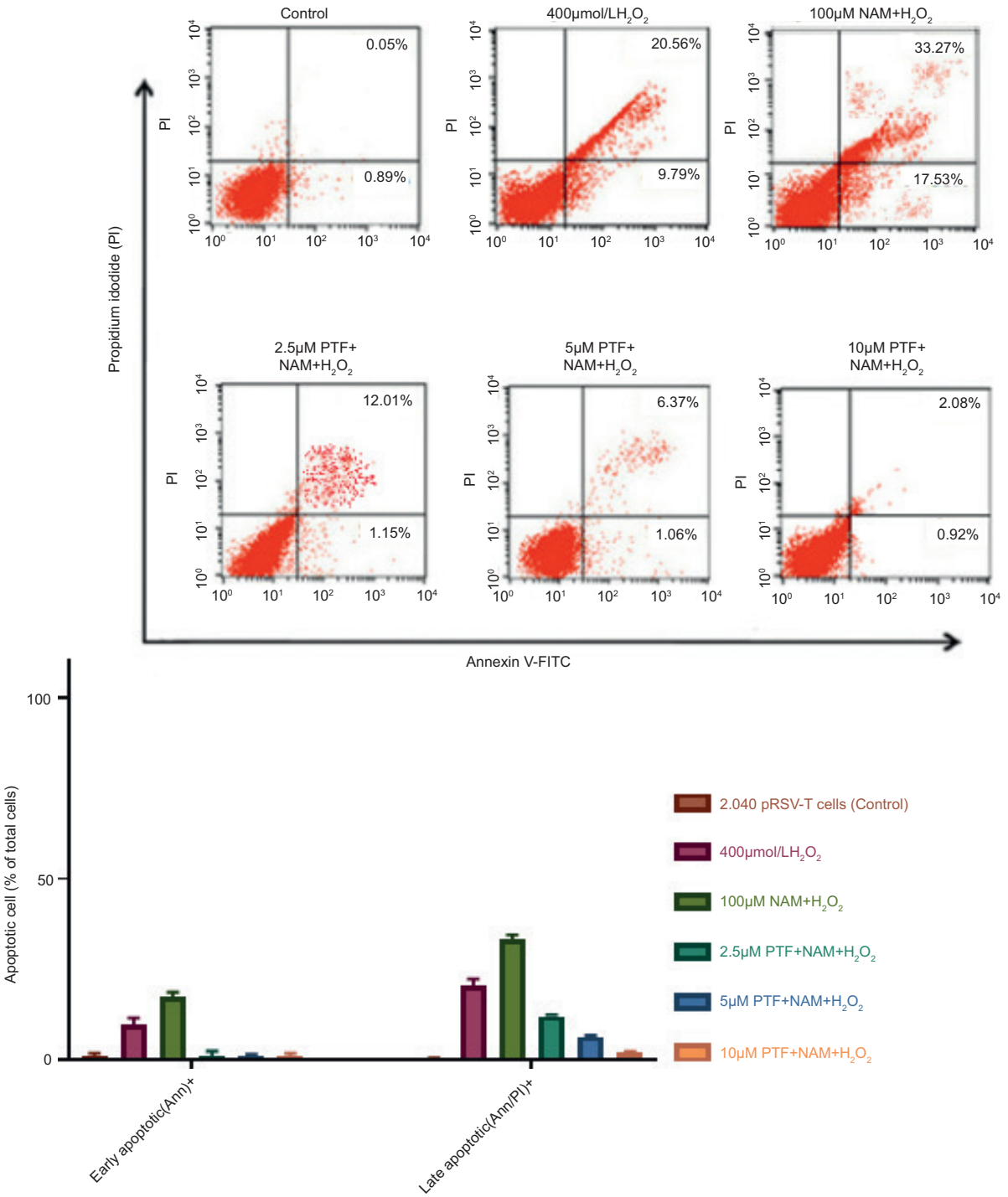

FIGURE 6. Effects of pifithrin-α (PFT-α), a p53 pathway inhibitor, on the percentage of apoptotic 2.040 pRSV-T cell lines with nicotinamide (NAM) and H_2O_2 treatments. The percentage of apoptotic cells increased significantly when NAM was added, while it decreased significantly when PFT-α concentration was increased. *Significant difference in comparison to cells treated with NAM and H_2O_2, $p < 0.001$.

J Popul Ther Clin Pharmacol Vol 29(3):e17–e33; 16 July 2022.

the control group, $p < 0.001$. However, when compared to the NAM and H_2O_2 groups, the percentage of apoptotic 2.040 pRSV-T cells decreased significantly by 10 μM PFT-α to reach about 2.9%. ($p < 0.001$, Figure 6).

DISCUSSION

The cornea is the principal refraction and transparency portion of the eye.[5] Because of its highly exposed location, the cornea is vulnerable to a number of environmental factors that lead to the formation of free radicals or ROS. The normal cornea contains both ROS and a detoxification system. Any defect in the equilibrium between the formation of ROS and the detoxification capability of the cornea results in oxidative damage and corneal diseases.[5] SIRT1 is an extremely preserved NAD^+ based histone deacetylase that belongs to the Sirtuin family (silent information regulator).[21] SIRT1 controls a variety of biological functions and disease conditions by eliminating the acetyl groups from different proteins. SIRT1 regulates the actions of many transcription factors and co-factors, influencing a subsequent expression of genes, and ultimately mitigating oxidative damage. SIRT1 malfunction has been associated with optical disorders such as cataracts, age-related macular degeneration (AMD), diabetic retinopathy (DR), and glaucoma, according to many investigations, while overexpression of SIRT1 protects against these conditions or disorders.[21] In the present study, the major finding is that the up-regulation of SIRT1 suppresses the apoptosis of normal corneal epithelial cells (2.040 pRSV-T cell line) with oxidative stress induced by H_2O_2. In particular, a study revealed that H_2O_2 can cause cell apoptosis through the p53 pathway.[22] While SIRT1 downregulation promotes apoptosis, this implies that SIRT1 protects the cornea against oxidative stress-induced apoptosis. Additionally, the results revealed that the etiology of SIRT1 protection involves p53 pathway inhibition. According to Western Blot examination of SIRT1 expression, SRT1720 produces a dose-dependent elevation in SIRT1 levels in H_2O_2-treated cultures (Figure 1). The results also revealed in current study that SIRT1 protects human corneal epithelial 2.040 pRSV-T cells from oxidative stress by blocking the p53 pathway. Apoptosis is triggered by the protein p53,[14,15] and it has been demonstrated that P53 is a SIRT1 substrate.[20,21] The p53 protein is involved in cellular responses to a wide range of stressors, including DNA damage, hypoxia, and oxidative stress. After physiological stressors, p53 becomes stabilized and attaches to DNA as a tetramer in a sequence-specific way, resulting in the transcriptional control of genes involved in important cellular processes such as cell-cycle arrest and apoptosis.[22,23] As illustrated in Figure 2, p53 expression was elevated in all H_2O_2 groups in comparison to the control group ($p< 0.0001$). A preceding study showed that H_2O_2 can cause apoptosis and cell death through the p53 pathway,[27] which is compatible with this study. The primary ubiquitination sites for p53 are found at its C terminal residues, and acetylation of these residues during periods of cell stress prevents protein breakdown and stabilizes p53.[28] Several acetylation enzymes hyperacetylate p53 in response to DNA damage and oxidative stress, stabilizing and stimulating P53 and causing it to initiate apoptosis.[26,14] On the other hand, SIRT1 produces deacetylation of p53 and suppresses p53-dependent apoptosis and transcriptional activity.[20,21] As a result, SIRT1 is essential in the anti-apoptosis mechanism, which has been reported in a number of earlier investigations.[27,28] In Figure 3.10, it was revealed that, in a dose-dependent manner, increasing the concentration of SIRT1720 (which is the most potent small molecule SIRT1 activator[32]) reduced acetyl-P53 levels under oxidative stress ($p< 0.005$), indicating that acetyl-p53 levels responded actively to the upregulation of SIRT1 expression and function under oxidative stress.

Apoptosis is a kind of programmed cell death that has been associated with cell loss. ROS, like H_2O_2, is a potent apoptotic agent that can cause cell

death. The results revealed that H_2O_2 at a high concentration (400μM) promotes cell apoptosis, consistent with earlier studies.[19,27] Flow cytometric analysis was used to differentiate between apoptotic and viable cells. As shown in Figure 4, 400μM H_2O_2 treatment significantly induced apoptosis in human corneal epithelial 2.040 pRSV-T cell lines via the P53 pathway (H_2O_2 at high concentrations activated P53, which induces apoptosis[19,22] because P53 is implicated in the cornea's stress response, and it mediates cell cycle arrest and apoptosis.[33] SRT1720 doses up to 7μM significantly reduced the percentage of apoptotic 2.040 pRSV-T cells by 10–4% ($p< 0.005$), in comparison with the H_2O_2 group. SRT1720 has been shown to reduce H_2O_2-induced apoptosis (Figure 4) in 2.040 pRSV-T human corneal epithelial cells by up-regulating the expression and enhancing the activity of SIRT1 that produces deacetylation and inhibits the activity of P53 and prevents human corneal epithelial cell apoptosis. SIRT1 is important in the anti-apoptosis pathway, which has been established in a number of prior investigations.[24,25] This means that the expression level of P53-dependent apoptosis was down regulated via overexpression of SIRT1. Pifithrin-α (PFT-α) is a specific p53 inhibitor that has been established to suppress p53-dependent apoptosis.[31] In comparison to the control group, the cell density of human corneal epithelial cells treated with 100μM NAM+H_2O_2 (Figure 5) was reduced, with significant alterations in shaped cells. This is due to the fact that H_2O_2 promotes cell apoptosis via the P53 pathway,[19,22] while NAM is the most potent and selective inhibitor of SIRT1 deacetylation activity[7,30] and causes down regulation of SIRT1 level and up-regulation of acetylated P53 that enhances human corneal epithelial cell apoptosis. P53's acetylation is crucial for its stability and function.[13] Interestingly, cell density was restored when 2.040 pRSV-T cell lines were treated with 5 and 10μM PFT-α in addition to NAM and H_2O_2 (Figure 5; $p< 0.05$). In a dose-dependent manner, PFT-α produced an elevation in cell density in comparison to the cells treated with NAM and H2O2. This is attributed to fact that PFT-α enhanced the level of SIRT1 protein,[32] that causes the deacetylation of P53-dependent apoptosis. In this experiment, the results demonstrated that blocking the p53 pathway via PFT-α could remove NAM's pro-apoptotic effect, which suppressed SIRT1 function and accelerated human corneal epithelial 2.040 pRSV-T cells' apoptosis. This indicates that SIRT1 is a negative regulator of P53 protein expression and has a protective effect via the downstream p53 pathway. The effect of PFT-α on the proportion of apoptotic corneal epithelial cells is evaluated in this experiment. When the 2.040 pRSV-T cells were treated with H_2O_2, the percentage of apoptotic cells was elevated by 30%, because H_2O_2 at high doses (400μM) activated P53 that is involved in apoptosis and cell death. When the cells were treated with NAM and H_2O_2, the percentage of apoptotic 2.040 pRSV-T cells increased by 20% in comparison to the control group ($p< 0.001$).

NAM is a potent physiological inhibitor of SIRT1, is a NAD^+ reaction product that inhibits SIRT1 non-competitively with $IC_{50} < 50$μM via negative feedback control of NAD^+.[35] NAM inactivates SIRT1 deacetylation function and stimulated acetylation of p53 results in an increased level of acetyl P53. This, together with H_2O_2 activated P53, promotes human corneal epithelial cell apoptosis and increases the percentage of apoptotic cells (Figure 6). PFT-α which is a specific inhibitor of p53 transcriptional activity[36] was revealed to significantly reduce the percentage of apoptotic cells in a dose-dependent manner. PFT-α inhibits P53-dependent apoptosis and removes NAM's pro-apoptotic effects, which suppress SIRT1 activity and increase apoptosis, resulting in SIRT1 upregulation, which produces more downstream of the P53 pathway and reduces the percentage of apoptotic normal corneal epithelial 2.040 pRSV-T cells. This strongly indicates that SIRT1 plays a significant role in protecting human corneal epithelial cells from oxidative stress through P53 pathway inhibition. SIRT1 has also been reported to protect many kinds of cells from apoptosis by blocking the p53

pathway, such as lens epithelial cells,[7] islet cells,[37,38] cardiac myocytes,[39] and osteoblasts in previous investigations.

CONCLUSIONS

This study revealed that SIRT1 regulates human corneal (2.040 pRSV-T) cells resistance to oxidative stress via p53 protein deacetylation and suppresses p53-dependent apoptosis. SIRT1 activators could potentially be used in the future to prevent oxidative damage in the cornea.

CONFLICTS OF INTEREST

There are no conflicts to declare.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Mustansiryiah University (www.uomustansiryiah.edu.iq), Baghdad, Iraq, for its support with this work.

REFERENCES

1. Goldstein EB. Sensation and perception. 8th International Edition. Belmont, CA (US): Wadsworth. 2010.
2. Mimura T, Kaji Y, Noma H, Funatsu H, Okamoto S. The role of SIRT1 in ocular aging. Exp Eye Res 2013 Nov;116:17–26. https://doi.org/10.1016/j.exer.2013.07.017
3. Wenk J, Brenneisen P, Meewes C, Wlaschek M, Peters T, Blaudschun R, et al. UV-induced oxidative stress and photoaging. Curr Probl in Dermatol 2001;29:83–94. https://doi.org/10.1159/000060656
4. Choi S-I, Dadakhujaev S, Ryu H, im Kim T, Kim EK. Melatonin protects against oxidative stress in granular corneal dystrophy type 2 corneal fibroblasts by mechanisms that involve membrane melatonin receptors. J Pineal Res 2011;51(1):94–103. https://doi.org/10.1111/j.1600-079X.2011.00866.x
5. Vallabh NA, Romano V, Willoughby CE. Mitochondrial dysfunction and oxidative stress in corneal disease. Mitochondrion 2017 Sep;36:103–13. https://doi.org/10.1016/j.mito.2017.05.009
6. Güllülü Ö, Hehlgans S, Rödel C, Fokas E, Rödel F. Tumor suppressor protein p53 and inhibitor of apoptosis proteins in colorectal cancer-a promising signaling network for therapeutic interventions. Cancers 2021 Jan;13(4):624. https://doi.org/10.3390/cancers13040624
7. Zheng T, Lu Y. SIRT1 protects human lens epithelial cells against oxidative stress by inhibiting p53-dependent apoptosis. Current Eye Res 2016 Aug 2;41(8):1068–75. https://doi.org/10.3109/02713683.2015.1093641
8. Dittenhafer-Reed KE, Feldman JL, Denu JM. Catalysis and mechanistic insights on Sirtuin activation. Chembiochem 2011 Jan 24;12(2):281–9. https://doi.org/10.1002/cbic.201000434
9. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature 2007 Nov;450(7170):712–6. https://doi.org/10.1038/nature06261
10. Tsai T-Y, Chen T-C, Wang I-J, Yeh C-Y, Su M-J, Chen R-H, et al. The effect of resveratrol on protecting corneal epithelial cells from cytotoxicity caused by moxifloxacin and benzalkonium chloride. Investig Ophthalmol Vis Sci 2015 Mar 5;56(3):1575–84. https://doi.org/10.1167/iovs.14-15708
11. Zeng L, Chen R, Liang F, Tsuchiya H, Murai H, Nakahashi T, Iwai K, Takahashi T, Kanda T, Morimoto S. Silent information regulator, Sirtuin 1, and age-related diseases. Geriatr gerontol int 2009 Mar;9(1):7–15. https://doi.org/10.1111/j.1447-0594.2008.00504.x
12. Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin in Cell Biol 2003 Apr 1;15(2): 164–71. https://doi.org/10.1016/S0955-0674(03)00003-6
13. Luo J, Nikolaev AY, Imai SI, Chen D, Su F, Shiloh A, Guarente L, Gu W. Negative control of p53 by Sir2α promotes cell survival under stress. Cell 2001 Oct 19;107(2):137–48. https://doi.org/10.1016/S0092-8674(01)00524-4

14. Yamamoto H, Schoonjans K, Auwerx J. Sirtuin functions in health and disease. J Mol Endocrinol 2007 Aug 1;21(8):1745–55. https://doi.org/10.1210/me.2007-0079
15. Al-Sudani B, H. Ragazzon-Smith A, Aziz A, Alansari R, Ferry N, Krstic-Demonacos M, et al. Circular and linear: a tale of aptamer selection for the activation of SIRT1 to induce death in cancer cells. RSC Adv 2020;10(73):45008–18. https://doi.org/10.1039/D0RA07857C
16. Tariq R, Arif I, Basma T, Al-Sudani, Al-Sudani B. SIRTUIN 1, a novel approach in the treatment of type 2 diabetes mellitus. Syst Rev Pharm 2020 Nov 1;11:117–28.
17. Mahmood T, Yang P-C. Western Blot: technique, theory, and trouble shooting. N Am J Med Sci 2012 Sep;4(9):429–34. https://doi.org/10.4103/1947-2714.100998
18. Telford W, Tamul K, Bradford J. Measurement and characterization of apoptosis by flow cytometry. Curr Protoc Cytom 2016 Jul;77(1):9–49. https://doi.org/10.1002/cpcy.1
19. Strober W. Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology 2015;111(1):A3.B.1–A3.B.3. https://doi.org/10.1002/0471142735.ima03bs111
20. Zhou M, Luo J, Zhang H. Role of Sirtuin 1 in the pathogenesis of ocular disease (Review). Int J Mol Med 2018 Aug 1;42(1):13–20. https://doi.org/10.3892/ijmm.2018.3623
21. Liu T, Ma X, Ouyang T, Chen H, Lin J, Liu J, et al. SIRT1 reverses senescence via enhancing autophagy and attenuates oxidative stress-induced apoptosis through promoting p53 degradation. Int J Biol Macromol 2018 Oct;117:225–34. https://doi.org/10.1016/j.ijbiomac.2018.05.174
22. Vazquez A, Bond EE, Levine AJ, Bond GL. The genetics of the p53 pathway, apoptosis and cancer therapy. Nat Rev Drug Discov 2008 Dec;7(12):979–87. https://doi.org/10.1038/nrd2656
23. Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 2008 May;9(5):402–12. https://doi.org/10.1038/nrm2395
24. Niwa-Kawakita M, Ferhi O, Soilihi H, Le Bras M, Lallemand-Breitenbach V, de Thé H. PML is a ROS sensor activating p53 upon oxidative stress. J Exp Med 2017 Nov 6;214(11):3197–206. https://doi.org/10.1084/jem.20160301
25. Brooks CL, Gu W. p53 Ubiquitination: Mdm2 and Beyond. Mol Cell 2006 Feb 3;21(3):307–15. https://doi.org/10.1016/j.molcel.2006.01.020
26. Lin Z, Yang H, Kong Q, Li J, Lee SM, Gao B, Dong H, Wei J, Song J, Zhang DD, Fang D. USP22 antagonizes p53 transcriptional activation by deubiquitinating Sirt1 to suppress cell apoptosis and is required for mouse embryonic development. Mol Cell. 2012 May 25;46(4):484–94. https://doi.org/10.1016/j.molcel.2012.03.024
27. Niederer F, Ospelt C, Brentano F, Hottiger MO, Gay RE, Gay S, Detmar M, Kyburz D. SIRT1 overexpression in the rheumatoid arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance. Ann Rheum Dis 2011 Oct 1;70(10):1866–73. https://doi.org/10.1136/ard.2010.148957
28. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem 2002 Nov 22;277(47):45099–107. https://doi.org/10.1074/jbc.M205670200
29. Liang J, Cao R, Wang X, Zhang Y, Wang P, Gao H, et al. Mitochondrial PKM2 regulates oxidative stress-induced apoptosis by stabilizing Bcl2. Cell Res 2017 Mar;27(3):329–51. https://doi.org/10.1038/cr.2016.159
30. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, Gudkov AV. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999 Sep 10;285(5434):1733–7. https://doi.org/10.1126/science.285.5434.1733
31. Villalba JM, Alcaín FJ. Sirtuin activators and inhibitors. BioFactors 2012;38(5):349–59. https://doi.org/10.1002/biof.1032
32. Liu C, Vojnovic D, Kochevar IE, Jurkunas UV. UV-A irradiation activates Nrf2-regulated antioxidant defense and induces p53/caspase3-dependent apoptosis in corneal endothelial cells. Inves Ophthalmol Vis Sci 2016 Apr 29;57(4):2319–27. https://doi.org/10.1167/iovs.16-19097

33. Wu J, Liang W, Tian Y, Ma F, Huang W, Jia Y, et al. Inhibition of P53/miR-34a improves diabetic endothelial dysfunction via activation of SIRT1. J Cell Mol Med. 2019;23(5):3538–48. https://doi.org/10.1111/jcmm.14253
34. Qiu Y, Zhou X, Liu Y, Tan S, Li Y. The role of sirtuin-1 in immune response and systemic lupus erythematosus. Front Immunol 2021 Apr 26;12:632383. https://doi.org/10.3389/fimmu.2021.632383
35. Zhu J, Singh M, Selivanova G, Peuget S. Pifithrin-α alters p53 post-translational modifications pattern and differentially inhibits p53 target genes. Scientific reports. 2020 Jan 23;10(1):1–9. https://doi.org/10.1038/s41598-020-58051-1
36. Beumer TL, Roepers-Gajadien HL, Gademan IS, Buul PP van, Gil-Gomez G, Rutgers DH, et al. The role of the tumor suppressor p53 in spermatogenesis. Cell Death Differ. 1998 Aug;5(8):669–77. https://doi.org/10.1038/sj.cdd.4400396
37. Imai S, Kiess W. Therapeutic potential of SIRT1 and NAMPT-mediated NAD biosynthesis in type 2 diabetes. Front Biosci. 2009 Jan 1;14:2983–95. https://doi.org/10.2741/3428
38. Doganay S, Borazan M, Iraz M, Cigremis Y. The effect of resveratrol in experimental cataract model formed by sodium selenite. Current Eye Res 2006 Jan 1;31(2):147–53. https://doi.org/10.1080/02713680500514685
39. Bäckesjö CM, Li Y, Lindgren U, Haldosén LA. Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. Cells Tissues Organs. 2009;189(1–4):93–7. https://doi.org/10.1159/000151744