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Original article

Resveratrol inhibits apoptosis by increase in the proportion of chondrocytes in the S phase of cell cycle in articular cartilage of ACLT plus Mmx rats

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ABSTRACT

The current study was aimed to investigate the effect of resveratrol on apoptosis inhibition in chondrocytes in ACLT plus Mmx rat model. TUNEL assay revealed a markedly higher level of apoptotic chondrocytes in the cartilage of untreated ACLT plus Mmx rats. The percentage of apoptotic chondrocytes was found to be 29.5 and 40.75%, respectively at 21 and 45 days. The percentage of apoptotic chondrocytes at 21 and 45 days in resveratrol (5 mg/kg) treated ACLT plus Mmx rats was found to be 13% and 2%, respectively. Real-time PCR analysis revealed that treatment of the ACLT plus Mmx rats with resveratrol for 45 days caused a significant increase in the expression of miR-18a compared to that in untreated rats. Flow cytometry and BrdUrd incorporation assay revealed that the proportion of chondrocytes in the S phase was increased to 51.4% in resveratrol treatment group compared to 25.3% in the untreated ACLT plus Mmx rats. Western blot analysis showed that treatment of the ACLT plus Mmx rats with resveratrol decreased the expression of ATM protein kinase and GFP protein without any effect on the expression of GFP-γ-tubulin in chondrocytes. In addition, resveratrol treatment also led to reduction in the activity of luciferase in the chondrocytes of ACLT plus Mmx rats. Resveratrol treatment of the ACLT plus Mmx rats decreases the expression level of ATM protein and checkpoint kinase 2 (CHK2) phosphorylation in chondrocytes. H2AX and 53BP1 phosphorylation was decreased in ACLT plus Mmx rats on treatment with resveratrol for 45 days. Immunofluorescence results revealed a markedly lower level of H2AX and 53BP1 nuclear foci in the chondrocytes of ACLT plus Mmx rats treated with resveratrol. Thus resveratrol treatment of the ACLT plus Mmx rats inhibits chondrocyte apoptosis and increases proportion of cells in the S phase of cell cycle which may be through the increase in expression of miR18a. A significant relation appears between resveratrol and miR-18a expression in the chondrocytes.

1. Introduction

Osteoarthritis is a degenerative joint disease characterized by the loss of chondrocyte function and degradation of extracellular matrix (ECM) (Loeser, 2009). Strength of the articular cartilage is maintained by the chondrocytes through the formation of ECM molecules (Roughley, 2001). Apoptosis of chondrocytes causes deficiency of cartilage matrix which results in destruction of matrix (Aigner and Kim, 2002). Cell apoptosis is caused by damage to DNA which results from breaks in the double stranded structure and it activates DNA damage response triggers. The factors leading to DNA damage include ionizing radiation, use of chemotherapy and cellular stress (reactive oxygen species) (Khanna and Jackson, 2001). DNA damage initiates a response pathways which either repairs damage or causes cells to undergo apoptosis (Harper and Elledge, 2007). Defect in genes (RFC5, DPB11, etc) responsible for sensing and repairing damage results in the development of various disorders (Jiri and Jiri, 2003). Ataxia-telangiectasia mutated (ATM) kinase which governs activation of homologous recombination repair pathway counteracts the harmful effect of DNA damage (Beucher et al., 2009; Chen et al., 2007). Activation of ATM causes phosphorylation of CHK2 which in turn

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activates CDC25A and CDC25C leading to cell cycle arrest in the S phase or G2/M phases, respectively (Matsuoka et al., 2000). H2AX and p53 binding protein 1 (53BP1) phosphorylation is also performed by ATM activation at the time of DNA damage which is evident by the appearance of discrete nuclear foci (Stiff et al., 2004; Adams and Carpenter, 2006). The target gene of miR-18a is ATM protein kinase. Increased expression of miR-18a causes reduction in ATM expression which leads to decrease in the repair of DNA and increase in chondrocyte proliferation. Therefore, chondrocyte apoptosis in the osteoarthritis rats can be related with the expression of miR-18a.

Resveratrol synthesized in the plants at the time of environmental stress and attack by pathogens plays an important role in the prevention of cardiovascular diseases (Ciolino et al., 1998). Studies have revealed that resveratrol can be used as a promising agent for the treatment of cancer, inflammation related diseases and heart disease (Martinez and Moreno, 2000). Lipoprotein oxidation, formation of eicosanoids and aggregation of platelets is also inhibited by resveratrol (Bertelli et al., 1995). It has also been found that resveratrol alters the activities of various enzymes linked with the replication of DNA and proliferation of cells (Fontecave et al., 2000). The current study was aimed to investigate the effect of resveratrol on the apoptosis inhibition in chondrocytes in ACLT plus Mmx rat model. The study showed that resveratrol treatment inhibited apoptosis induction in the articular cartilage chondrocytes in ACLT plus Mmx rats.

2. Materials and methods

2.1. Animals

Twenty healthy adult male Sprague-Dawley rats (~170–210 g weight) were obtained from the Experimental Animal Centre of China Medical University (Shenyang, China). The rats were housed under 12 h light and dark cycle at a temperature 25 °C and ~60% humidity. The animal were provided free access to food and water.

2.2. Ethical statement

The Committee on Animal Care and Use of China Medical University approved all the experiments and procedures for the animals. The animal experimental protocols were given approval by the Ethics Committee of the School of Medicine, China Medical University, under the NIH guidelines for the ethical care of experimental animals.

2.3. Animal model preparation and treatment

The animals were given halothane anesthesia, shaved and then disinfected. Medial para-patellar method was used for the exposure of right knee joint and subsequently the lateral dislocation of the patella was performed. Knee joint was put in complete flexion and subsequently micro-scissors were used for anterior cruciate ligament transection and medial meniscus resection (ACLT plus MMx). In the normal control group exposure of the knee joint and patellar subluxation was followed by saline washing closure of incisions. The animals were divided into three groups; normal control, untreated ACLT plus MMx and the treatment ACLT plus MMx groups. The rats in the treatment group were administered intragastrically with 5 mg/kg resveratrol daily various durations (7–45 days). The animals in the normal control and untreated control groups received only normal saline.

2.4. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)

Analysis of chondrocyte apoptosis in the cartilage specimens was performed in situ by employing cell apoptosis detection kits (Boster Biological Technology, Ltd., Wuhan, China) in accordance with manual protocol. Rats after resveratrol treatment or untreated were sacrificed at the time indicated in the results section. Tibia and femur bones were extracted, dissected and subsequently subjected to paraformaldehyde fixing for 24 h. The bones were treated with ethylenediamine tetraacetic acid (10%) for decalcification followed by paraffin embedding and sectioning into 4 μm sections. Deparaffinization of the sections and was followed by permeabilization using Triton X-100 (0.1%). Fluorescein-labeled dUTP and terminal deoxynucleotidyl transferase (TdT) mixture was used for the incubation of the sections. Probing and development of the sections was performed using antifluorescein antibody conjugated with alkaline phosphatase and fast red, respectively. The Olympus OX31 microscope (Olympus, Tokyo, Japan) and Image-Pro Plus 6.0 System were used for calculation of apoptosis index and quantitative analysis, respectively.

2.5. RNA extraction, reverse transcription (RT) and real-time PCR

The mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) was used for the isolation of total miRNA from the chondrocytes according to the manual protocol. From the extracted RNA 2 μg samples were used for the synthesis of cDNA by employing the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA). The miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) was used for quantification of miR-18a expression levels. The Applied Biosystems 7500 Sequence Detection system was used for the purpose of Real-time PCR.

2.6. Bromodeoxyuridine labeling

Analysis of the checkpoint for S-phase in the chondrocytes was performed using coverslips (Fisher Scientific). The chondrocytes were exposed to radiations and subsequently subjected to incubation incubated for 24 h. The chondrocytes were treated with BrdUrd and then incubation was continued for 2 h more. After incubation the chondrocytes were subjected to fixing and staining using anti-BrdUrd antibody (Upstate, Temecula, CA) in accordance with the manufacturer's procedure. The laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany) was used for acquiring the gray level images.

2.7. Immuno fluorescence

Chondrocytes were fixed for 15 min using ice-cold methyl alcohol on the coverslips (Fisher Scientific) followed by treatment with 10% goat serum in PBS. The chondrocytes were subjected to incubation in goat serum/PBS (10%) with anti-bodies against H2AX (Abcam) and 53BP1 (Bethyl, Inc.). Rhodamine-conjugated goat anti-rabbit IgG (The Jackson Laboratory) was used for the detection of primary antibodies and DAPI was employed for the staining of the DNA. The laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany) was used for acquiring the level of gray images.

2.8. Analysis of chondrocyte apoptosis using flow cytometry

The proportion of chondrocytes undergoing apoptosis was determined using Annexin V-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China). The chondrocytes after washing twice with PBS were treated with binding buffer. The 100 ml of the chondrocyte suspension was then put into culture

tube (5 ml) and treated with Annexin V-FITC (5 μl) and propidium iodide (10 μl). Incubation of the culture tube was performed for 20 min under dark conditions. To each of the tube was added 400 ml of the binding buffer for flow cytometry analysis using flow cytometer (FACSCalibur; BD Biosciences).

2.9. Cell cycle analysis

The chondrocytes were collected, washed twice with PBS for 10 min at 1200 rpm. The chondrocytes were then subjected to fixing using 70% ethyl alcohol and subsequently washed with PBS. Staining of the chondrocytes was performed with propidium iodide for 45 min in the presence of RNase A (100 μl) followed by flow cytometry analysis.

2.10. Western blot analysis

The 20 μg protein samples were subjected to electrophoresis by loading onto 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel. After separation electro blotting apparatus (Bio-Rad, Richmond, CA) was used to transfer the proteins onto a nitrocellulose membrane. Incubation of the membranes was performed with primary antibodies against ATM, CHK2, p-CHK2, 53BP1, p-53BP1 (Cell Signaling, Danvers, MA), ϒ-H2AX (Abcam, Cambridge, MA), antiHA (Sigma, Saint Louis, MI). TBS-T and HRP-conjugated secondary anti-rabbit antibody were used for the washing and incubation of the blots. ECL reaction system and LAS-3000 Luminescent Image Analyzer (FujiFilm, Japan) were used for developing and visualization of the blots.

2.11. Statistical analysis

The expressed data are the mean ± standard deviation. Analysis of the data was performed using two-tailed Student's *t*-test, within the MS Excel 2007 software package (Microsoft Corp., Redmond, WA, USA). The statistically significant values were considered different if $P < 0.05$.

3. Results

3.1. Effects of resveratrol on chondrocyte apoptosis in the knee joints of ACLT plus Mmx rats

TUNEL assay revealed a markedly higher level of apoptotic chondrocytes in the cartilage of untreated ACLT plus Mmx rats compared to the normal control group. The percentage of apoptotic chondrocytes in the cartilage of untreated ACLT plus Mmx rats was found to be 29.5 and 40.75%, respectively at 21 and 45 days. In the ACLT plus Mmx rats, resveratrol (5 mg/kg) treatment significantly decreased the percentage of apoptotic chondrocytes at 21 and 45 days. The percentage of apoptotic chondrocytes at 21 and 45 days after resveratrol treatment in the ACLT plus Mmx rats was found to be 13% and 2%, respectively (Fig. 1).

3.2. Resveratrol upregulates the expression of miR-18a in the chondrocytes of ACLT plus Mmx rats

The expression of miR-18a in the chondrocytes of articular cartilage in the knee joints of ACLT plus Mmx rats was found to be markedly lower as revealed by Real-time PCR analyses. However, treatment of the ACLT plus Mmx rats with resveratrol for 45 days caused a significant increase in the expression of miR-18a in the chondrocytes of articular cartilage compared to that in untreated rats (Fig. 2).

3.3. Resveratrol alters cell cycle distribution in the chondrocytes of ACLT plus Mmx rats

Flow cytometry revealed that treatment of the ACLT plus Mmx rats with resveratrol for 45 days caused a significant increase in the accumulation of chondrocytes of articular cartilage in the S phase compared with that in the untreated ACLT plus Mmx rats. The proportion of chondrocytes in the S phase was increased to 51.4% in resveratrol treatment group compared to 25.3% in the untreated ACLT plus Mmx rats (Fig. 3A). BrdUrd incorporation assay also showed a marked increase in the S-phase fraction in the chondrocytes of ACLT plus rats (51.4%) on treatment with resveratrol compared to that in untreated rats (23.8%) (Fig. 3B).

3.4. Resveratrol treatment causes reduction in the expression of ATM

Western blot analysis showed that treatment of the ACLT plus Mmx rats with resveratrol decreased the expression of ATM protein kinase. Resveratrol treatment of the of ACLT plus Mmx rats caused a marked decrease in the expression of GFP protein without any effect on the expression of GFP-γ-tubulin in chondrocytes (Fig. 4A). In addition, resveratrol treatment also led to reduction in the activity of luciferase in the chondrocytes of ACLT plus Mmx rats (Fig. 4B).

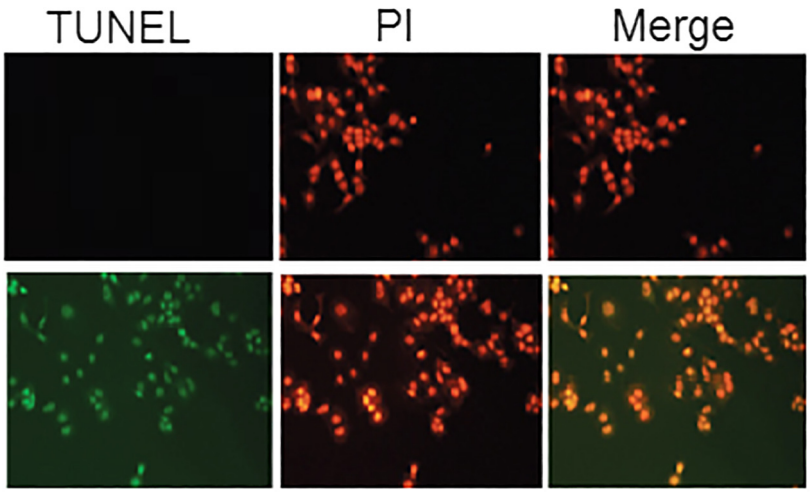

Fig. 1. Resveratrol treatment decreases the percentage of apoptotic chondrocytes in the ACLT plus Mmx rats. The rats were treated with 5 mg/kg doses of resveratrol for 45 days and then the population of apoptotic chondrocytes was determined using TUNEL assay.

3.5. Resveratrol causes the ATM signaling pathway impairment

Resveratrol treatment of the ACLT plus Mmx rats decreases the expression level of ATM protein and checkpoint kinase 2 (CHK2) phosphorylation in chondrocytes. H2AX and 53BP1 phosphorylation was decreased in ACLT plus Mmx rats on treatment with resveratrol for 45 days (Fig. 5A). Immunofluorescence results revealed a markedly lower level of H2AX and 53BP1 nuclear foci in the chondrocytes of ACLT plus Mmx rats treated with resveratrol (Fig. 5B).

4. Discussion

Osteoarthritis a joint disease characterized by bone degeneration is caused by various factors with diverse mechanisms underlying. Apoptosis of chondrocytes and degeneration of the ECM plays an important role in the osteoarthritis development and progression. The current study was aimed to investigate the effect of resveratrol on inhibition of chondrocyte apoptosis in ACLT plus MMx rat model. The study demonstrated that resveratrol inhibits apoptosis of articular cartilage in ACLT plus MMx rats probably

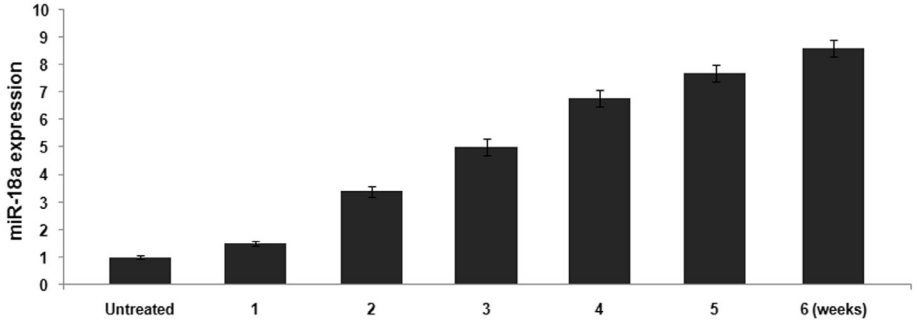

Fig. 2. Resveratrol up-regulates the expression of miR-18a in the chondrocytes of articular cartilage in ACLT plus Mmx rats. Real-time PCR analysis showed that resveratrol treatment for 45 days increased the expression of miR-18a in the chondrocytes. $P < 0.005$ compared with the normal control group.

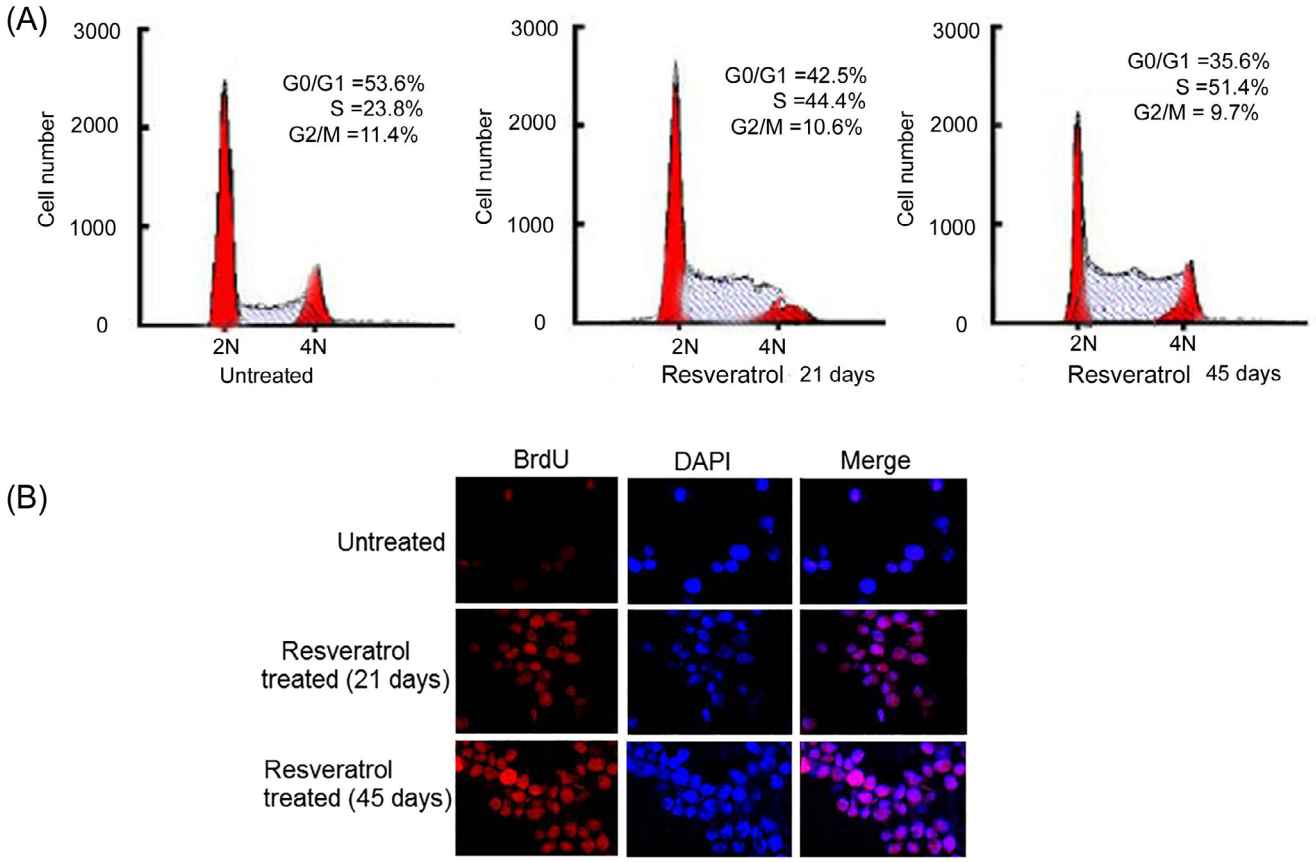

Fig. 3. Resveratrol treatment increased S phase chondrocyte population. (A) Flow cytometric analysis showed that resveratrol increased the proportion of chondrocytes in the S phase. The cell cycle distribution was analyzed in the chondrocytes of ACLT plus Mmx rats after 45 days of treatment with 5 mg/kg doses of resveratrol. (B) BrdUrd incorporation assay was used to analyze the population of chondrocytes in the S phase of cell cycle.

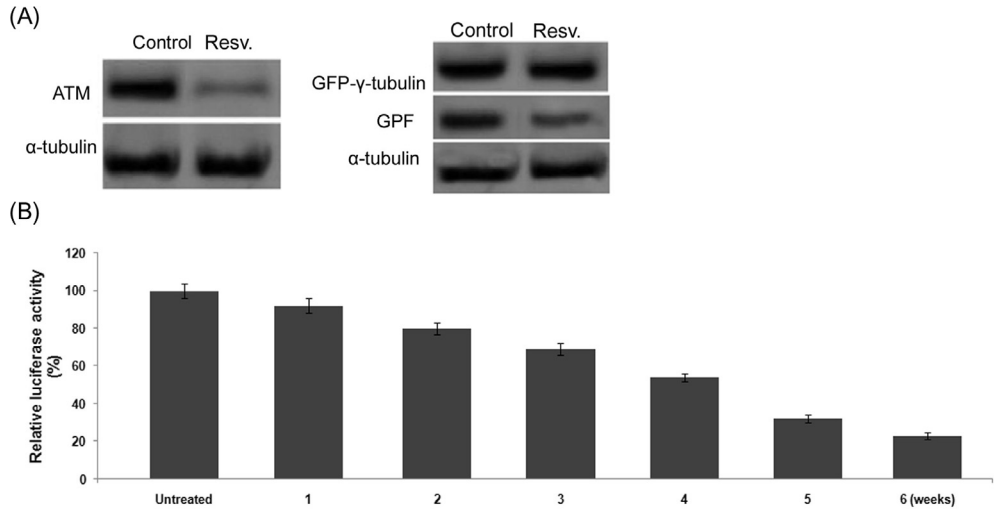

Fig. 4. (A) Western blot analysis showed that resveratrol treatment decreased the expression of ATM and GFP protein without any effect on the expression of GFP-γ–tubulin. The expression of ATM, GFP and GFP-γ–tubulin in the chondrocytes of ACLT plus Mmx rats was analyzed after 45 days of resveratrol treatment. (B) Luciferase activity analysis also showed reduction in the chondrocytes of ACLT plus Mmx rats after 45 days of resveratrol treatment.

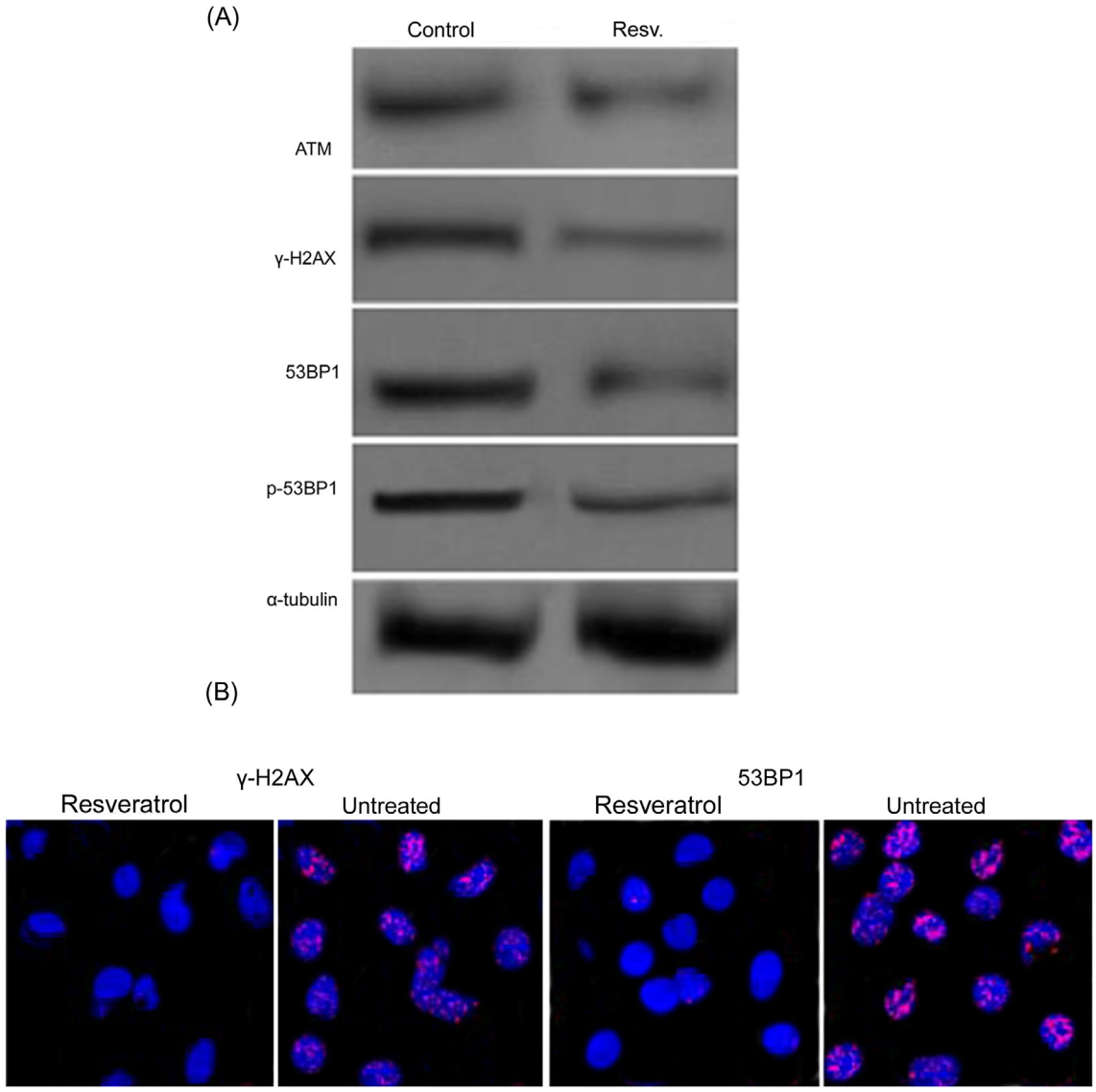

Fig. 5. Resveratrol treatment of ACLT plus Mmx rats interferes with ATM signaling. (A) Western blot assay showed reduction in the ATM expression and 53BP1 and phosphorylation of and checkpoint kinase 2 (CHK2) in the chondrocytes of ACLT plus Mmx rats after 45 days of resveratrol treatment. (B) Immunofluorescence showed lower H2AX and 53BP1 nuclear foci in the chondrocytes of ACLT plus Mmx rats treated with resveratrol.

by up-regulating the expression of miR-18a and increasing the proportion of chondrocytes in S phase of cell cycle. Cellular apoptosis is induced by various agents such as ionizing radiation, chemical treatment, onset of endogenous and mechanical stress leading to DNA damage with breaks in the double stranded structure (Lees-Miller and Meek, 2003; Khanna and Jackson, 2001). In the present study a markedly higher level of apoptotic chondrocytes in the cartilage of ACLT plus Mmx rats was observed. However, resveratrol (5 mg/kg) treatment significantly decreased the percentage of apoptotic chondrocytes at 21 and 45 days in the ACLT plus Mmx rats. One of the members of miR cluster oncomir-1 encoding 5 miRNAs is the miR-18a and the presence of the members of this cluster is reported in various types of tumors (Lawrie, 2007; Libing et al., 2011). Studies have demonstrated that miR18a plays an important role in the reduction of DNA damage repair signaling (Lees-Miller and Meek, 2003). Our results revealed that the expression of miR-18a in the chondrocytes of articular cartilage in the knee joints of ACLT plus Mmx rats was found to be markedly lower. However, treatment of the ACLT plus Mmx rats with resveratrol for 45 days caused a significant increase in the expression of miR-18a in the chondrocytes of articular cartilage compared to that in untreated rats. Treatment of the ACLT plus Mmx rats with resveratrol for 45 days caused a significant increase in the accumulation of chondrocytes of articular cartilage in the S phase compared with that in the untreated ACLT plus Mmx rats.

There are reports that DNA damage in the cells causes increase in the expression level of ATM (Berkovich and Ginsberg, 2003). Our results showed that the resveratrol treatment of the ACLT plus Mmx rats with led to decrease in the expression of ATM protein kinase. Resveratrol treatment of the ACLT plus Mmx rats caused a marked decrease in the expression of GFP protein without any effect on the expression of GFP-c-tubulin in the chondrocytes. In addition, resveratrol treatment also led to reduction in the activity of luciferase in the chondrocytes of ACLT plus Mmx rats. Resveratrol treatment of the ACLT plus Mmx rats decreasesd the expression level of ATM protein and checkpoint kinase 2 (CHK2) phosphorylation in chondrocytes. H2AX and 53BP1 phosphorylation was decreased in ACLT plus Mmx rats on treatment with resveratrol for 45 days.

Thus resveratrol treatment of the ACLT plus Mmx rats inhibits chondrocyte apoptosis and increases proportion of cells in the S phase of cell cycle which may be through the increase in expression of miR18a. A significant relation appears between resveratrol and miR-18a expression in the chondrocytes.

Conflict of interest

The authors declare no conflict of interest.

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