Icariin induces apoptosis in mouse MLTC-10 Leydig tumor cells through activation of the mitochondrial pathway and down-regulation of the expression of piwil4

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Abstract. The Leydig cell tumor, derived from interstitial cells, is a rare neoplasm. In most cases, Leydig cell tumors are benign, however, if the tumor is malignant, no effective treatments are currently available. In this study, we aimed to evaluate the effects of icariin on the growth of the mouse Leydig tumor cell line MLTC-1 and to examine its underlying mechanism. Icariin caused a dose-dependent decrease in the viability of MLTC-1 cells, which coincided with an increase in cell apoptosis through regulation of the expression of Bcl-2/Bax and cytochrome c, activation of caspase-9 and -3. Moreover, the pro-apoptotic effect of icariin on MLTC-1 cells is related to piwil4, since icariin induced a decrease in piwil4 protein expression and piwil4 silencing significantly enhanced the cytotoxic effects of icariin in MLTC-1 cells. These findings suggest a novel anticancer effect of icariin in Leydig cell tumor through activation of the mitochondrial pathway and down-regulation of the expression of piwil4.

Introduction

Testicular neoplasms include germ cell tumors and sex cord-stromal tumors. Germ cell tumors comprise 95% of all testicular neoplasms, while the remaining 5% of testicular tumors are sex cord-stromal tumors, which are derived from somatic cells, Sertoli and Leydig cells. The Leydig cell tumor is classified as an interstitial cell tumor, which are commonly benign; however, in adults, ~10% of cases are malignant (1-4). The etiology of Leydig cell tumor in humans remains unknown. Currently, in most cases, the standard therapy for benign Leydig cell tumors is considered to be orchiectomy. Recently, by retrospectively analyzing the long-term follow-up of a series of patients with Leydig cell tumors electively treated with testis sparing surgery, Glannarin *et al* (5) demonstrated that testis sparing surgery with frozen section examination has an excellent long-term oncological outcome. Therefore, testis-sparing surgery may be chosen for treating benign Leydig cell tumors to maintain fertility, especially in boys and young men (5-9). If the tumor is malignant, orchiectomy with retroperitoneal lymph node dissection is recommended, since chemotherapy and radiation therapy show limited efficacy in the treatment of malignant Leydig cell tumors. Survival time of patients with this malignancy range from 2 months to 17 years, and the mean survival time is 2 years (10,11). To improve the survival time of patients with malignant Leydig cell tumor, and to decrease the serious consequences, such as testicular dysfunction and infertility that is caused by the current treatment for benign Leydig cell tumors, alternative treatments need to be explored.

Icariin ($C_{33}H_{40}O_{15}$; molecular weight, 676.67), a flavonoid isolated from *Herba Epimedii*, is considered to be the main active component responsible for the actions of *Herba Epimedii*. Many studies have shown that it has a wide range of pharmacological and biological activities on endocrine, cardiovascular, genital, bronchial, urinary and immune systems (12-15). *In vitro* studies have demonstrated that icariin possesses antitumor activity (16,17). Here we evaluated the effects of icariin on the growth of the mouse Leydig tumor cell line, MLTC-1 and examined its underlying mechanism.

Materials and methods

Materials. Icariin (purity >98%) was purchased from China's National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from HyClone. Caspase-3, -8 and -9 detection kit, the Annexin V apoptosis detection kit, the protein extraction kit and the BeyoECL Plus Western blotting detection reagent were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP and Lipofectamine 2000 were purchased from Invitrogen (USA). Anti-piwil4 (sc-67593), caspase-9 and -3 inhibitor (DEVD-CHO and z-LEHD-fmk) were purchased from

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Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2, anti-Bax, anti-cytochrome c and anti-β-actin antibodies were purchased from Biosis (Beijing, China).

Cell culture. MLTC-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All the cells were placed in an incubator, containing 95% air and 5% CO_2 at 37˚C, and the media were replaced every 2 days.

MTT assay. The activity of icariin on cell proliferation was assesed by the MTT assay. In brief, MLTC-1 cells were plated in 96-multiwell plates at a density of $5x10^4$ cells/well. After adherent, icariin at indicated doses was added to the wells and incubated for 24 or 48 h. At the indicated time after the treatment, the supernatant was removed and an MTT solution (5 mg/ml final concentration) was added. After 4 h incubation at 37˚C, the MTT solution was removed and formazan was dissolved in DMSO. MTT reduction in living cells was quantified at a 550 nm wavelength using a Sunrise Remote Microplate Reader (Grodig, Austria). Survival of control groups was defined as 100% surviving, and the number of living cells in the treated groups was expressed as a percentage of the control groups.

Flow cytometry for cell apoptosis analysis. MLTC-1 cells were treated with control medium or 50 μg/ml icariin for 24 or 48 h, then the cells were harvested for the Annexin V assay using the Annexin V Apoptosis Detection Kit. Briefly, treated cells were centrifuged for 10 min to remove the media. Then the cells were rinsed with 1X binding buffer supplied by the manufacturer. The rinsed cells were resuspended in 200 μl of binding buffer. Subsequently, 5 μl of Annexin V and 10 μl of PI were added and incubated at room temperature for 15 min. Flow cytometric analysis was carried out using using a FACSVantage SE flow cytometer (Becton-Dickinson, USA).

Flow cytometric analysis of the cell cycle. To investigate the possible roles of icariin on the cell cycle, flow cytometric analysis was used. Cells ($1x10^6$) were trypsinized and centrifuged. The cell pellets were washed with PBS and fixed in 70% ethanol. Then MLTC-1 cells were centrifuged for 5 min and washed with 1X PBS, following a 30-min incubation with Hanks-buffered saline solution containing PI (10 μg/ml) and RNase (100 μg/ml) at room temperature. The cells were quantified on a FACSVantage SE flow cytometer (Becton-Dickinson).

Measurement of caspase-3, -8, and -9 activity. The activities of caspase-3, -8 and -9 were measured according to the manufacturer's instructions. In brief, MLTC-1 cells were removed from culture dishes, washed twice with PBS, and pelleted by centrifugation. Cell pellets were then treated for 10 min with iced lysis buffer. The suspensions were then centrifuged at 16000 x g for 15 min, and the supernatants were collected. Subsequently, the caspase substrates (Ac-DEVD-*p*NA for caspase-3, Ac-IETD-*p*NA for caspase-8 and Ac-LEHD-*p*NA for caspase-9) were added and the tubes were incubated at 37˚C for 2 h. During incubation, the caspases cleaved the substrates to form *p*NA, and the release of *p*NA was determined using the absorbance at 405 nm. The caspase-3, -8 and -9 activities were expressed as the percentage compared to control.

Western blot analysis. Briefly, for protein isolation, MLTC-1 cells were lysed with RIPA buffer. Insoluble material was removed by centrifugation at 12000 x g for 10 min at 4˚C and supernatants were collected. The protein concentration was determined by the BCA assay. Proteins were separated by SDS-PAGE on a 12% polyacrylamide and were transferred to a PVDF membrane, followed by antibody incubation. After washing three times, membranes were incubated with a secondary IgG antibody for 2 h and washed again. The antigen-antibody complex was detected using an Beyo-ECL kit following the manufacturer's protocol.

The effect of piwil4 on icariin-induced cell apoptosis. To evaluate the effect of piwil4 on icariin-induced cell apoptosis, the BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP was employed to down-regulate the expression of piwil4 in MLTC-1 cells. The sequence of oligonucleotides encoding the miRNA for piwil4 RNAi were as follows, top: 5'-TGCTGTAGTAATCCACA TATGTGACCGTTTTGGCCACTGACTGACGGTCAC ATGTGGATTACTA-3', bottom: 5'-CCTGTAGTAATCCAC ATGTGACCGTCAGTCAGTGGCCAAAACGGTCAC ATATGTGGATTACTAC-3'. Construction of the piwil4-pcDNA™ 6.2-GW/EmGFP miR vector and screening of the resistant colonies were performed according to manufacturer's procedure.

Subsequently, MLTC-1-NC cells (transfected with negative control vector) and MLTC-1-piwil4-RNAi (transfected with piwil4 miR RNAi vector) were treated without or with 50 μM icariin for 24 h, followed by the MTT test and cell apoptosis analysis as described above.

Statistical analysis. Data are expressed as the mean ± SD. Statistical differences were evaluated by using ANOVA. The level for a statistically significant difference was set at $P<0.05$.

Results

Effects of icariin on growth of MLTC-1 cells. To assess the anti-proliferative activity of icariin, MLTC-1 cells were treated with 12.5, 25, 50 and 100 μg/ml of icariin for 24 or 48 h. The MTT assay results demonstrated that 12.5 μg/ml icariin did not signficantly affect cell viability ($P>0.05$), while at concentrations above 12.5 μg/ml, the cell viability of MLTC-1 cells was inhibited in a time- and dose-dependent manner. As shown in Fig. 1, 24-h treatment with 25, 50 and 100 μg/ml icariin significantly reduced cell viability to 82.20±3.89% ($P<0.05$), 67.79±5.29% ($P<0.01$) and 55.93±6.72% ($P<0.01$), respectively. Treatment with 25, 50 and 100 μg/ml icariin after 48 h caused a reduction in cell viability to 65.93±8.72% ($P<0.01$), 48.90±5.30% ($P<0.01$) and 30.77±6.24% ($P<0.01$), respectively. The effective icariin concentration for 50% inhibition (EC50) of the MLTC-1 cell viability after 48 h was 50 μg/ml, which was thus the concentration chosen for the subsequent experiments.

Icariin treatment (50 μg/ml) induces apoptosis in MLTC-1 cells. To quantitatively assess the effects of 50 μg/ml icariin on MLTC-1 cells, MLTC-1 cells were treated with medium (control) or with 50 μg/ml icariin for 24 or 48 h. PI vs. Annexin V-FITC

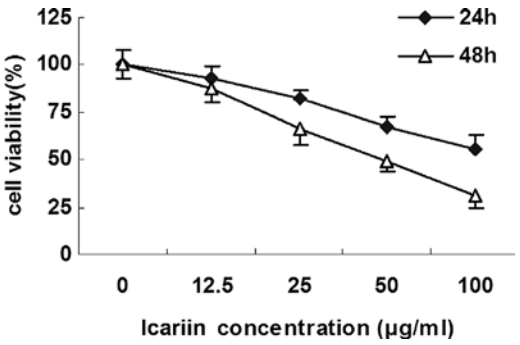

Figure 1. Inhibitory effects of icariin on MLTC-1 cell viability. MLTC-1 cells were treated with 12.5, 25, 50 and 100 μg/ml icariin for 24 or 48 h. At the end of the incubation period, the cell viability was estimated by MTT assay. The results are from three experiments and are presented as means ± SD.

fluorescence staining was then measured by flow cytometric analysis (Fig. 2). In the 24 h (Fig. 2A) and 48 h (Fig. 2C) vehicle-treated control group, 7.6±0.6% and 8.2±0.56% cells excluded PI and were positive for Annexin V-FITC binding, which represent the apoptotic cells. After exposure to 50 μg/ml icariin for 24 h (Fig. 2B) and 48 h (Fig. 2D), the percentage of apoptosis increased to 17.1±1.5% ($P<0.01$) and 24.7±1.6% ($P<0.01$), respectively.

Icariin affects the cell cycle progression of MLTC-1 cells. We determined the cell cycle phases in the cells with or without 50 μg/ml icariin treatment for 24 or 48 h by flow cytometric analysis (Fig. 3). The percentage of cells in the G_1 and G_2 phase after 24 or 48 h of icariin treatment was similar to that of the control groups ($P>0.05$). However, compared with

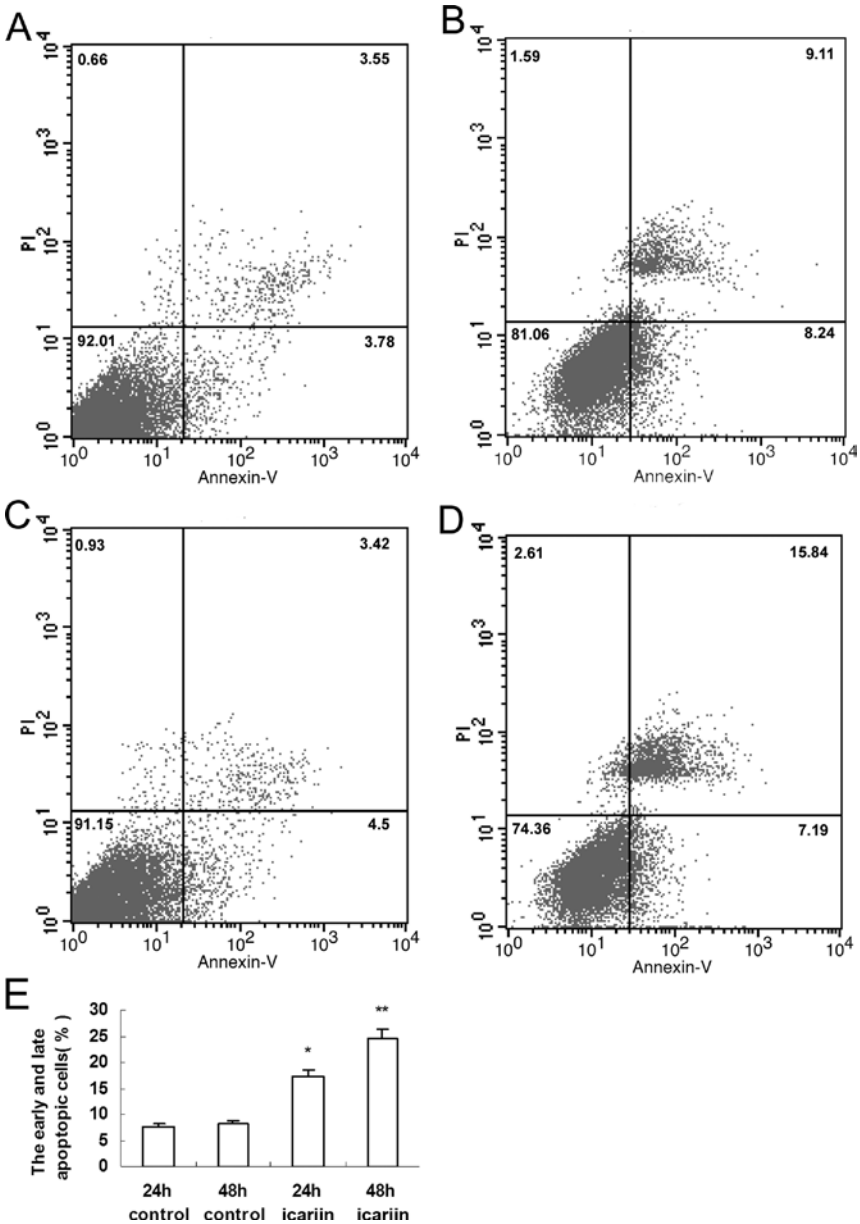

Figure 2. Treatment of MLTC-1 Leydig cells with 50 μg/ml icariin triggered apoptosis detected by the Annexin V/PI double-staining assay. The figures show representative flow cytometric histograms of cells treated with different experimental conditions. (A) The 24 h control group; (B) cells exposed to 50 μg/ml icariin for 24 h; (C) the 48 h control group; (D) cells exposed to 50 μg/ml icariin for 48 h. (E) The percentage of apoptotic cells for each treatment group. Values are the means ± SD (n=3). $^{*}P<0.01$, compared to the 24 h control group; $^{**}P<0.01$, compared to the 48 h control group.

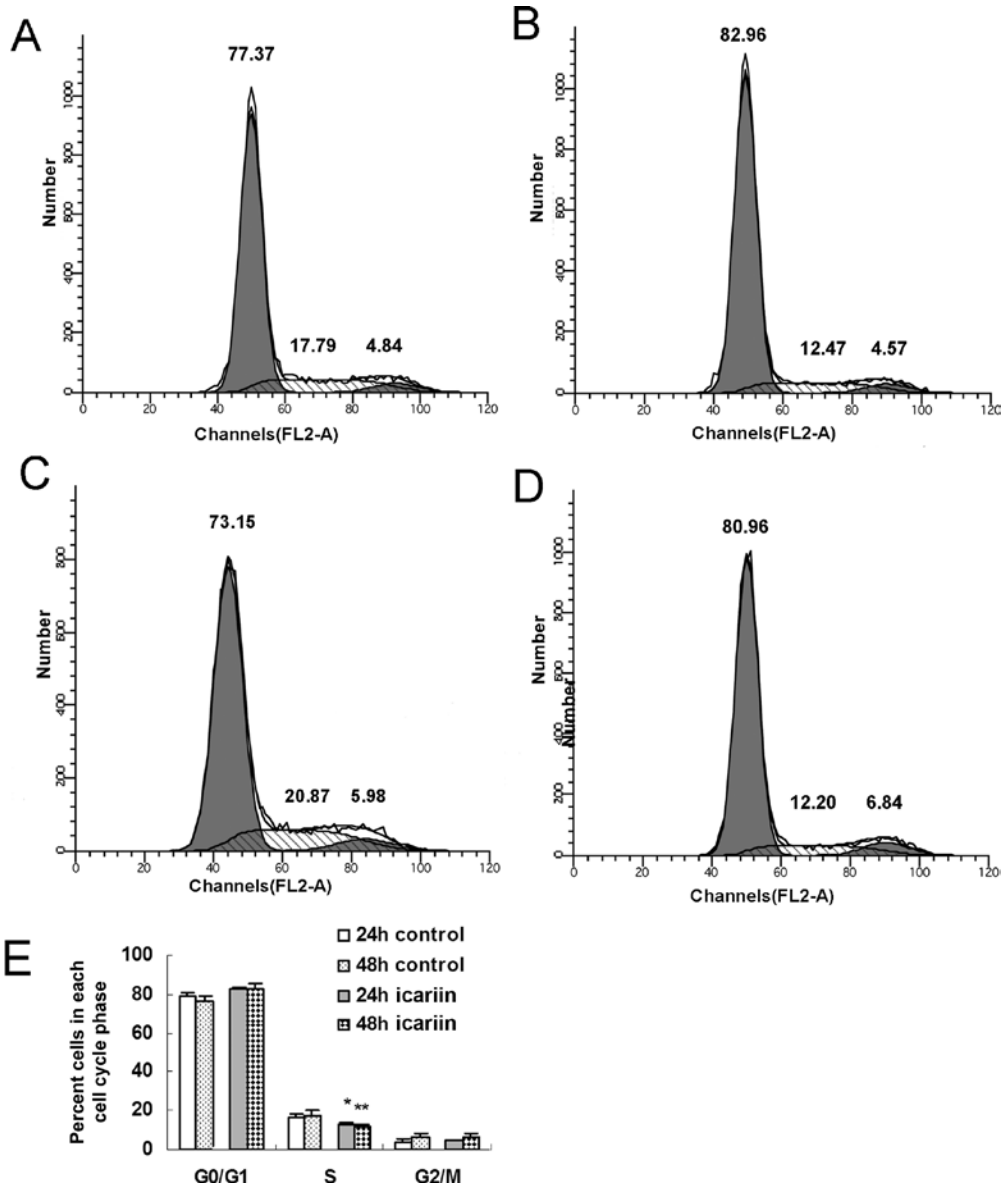

Figure 3. Effect of icariin on the cell cycle distribution of MLTC-1 cells. Representative flow cytometric analysis plot of the (A) 24-h control, (B) 50 μg/ml icariin for 24 h, (C) 48-h control and (D) 50 μg/ml icariin for 48 h treatment groups. Cells were fixed with 70% ethanol and stained with PI, followed by flow cytometric analysis. (E) Percentage of cells in the indicated phases of the cell cycle after 50 μg/ml icariin-treatment as determined by flow cytometry. The data are expressed as the means ± SD (n=3). *P<0.05, compared to the 24-h control group; **P<0.05, compared to the 48-h control group.

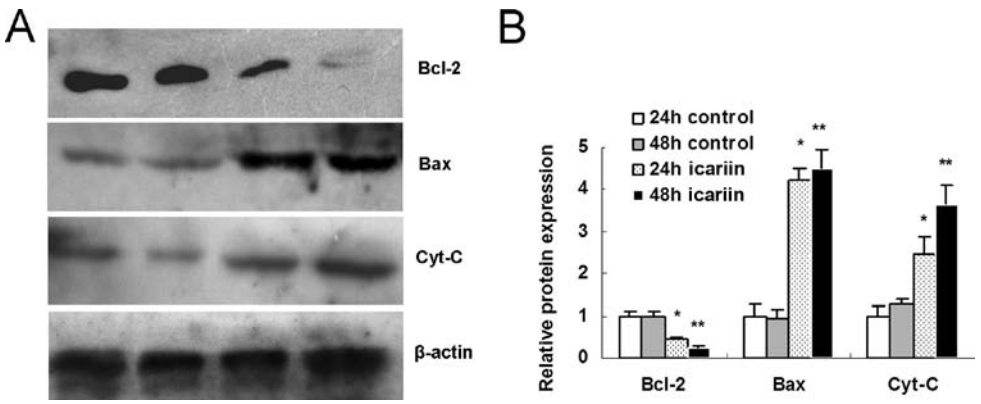

Figure 4. The expression of Bcl-2, Bax and cytochrome c (Cyt-C) in icariin-stimulated MLTC-1 cells. Cells were treated without or with 50 μg/ml icariin for 24 and 48 h, respectively. Bcl-2, Bax and cytochrome c-specific bands were detected by Western blotting. (A) Representative Western blotting of Bcl-2, Bax and cytochrome c. (B) Relative expression of Bcl-2, Bax and cytochrome c in different groups by normalizing to β-actin protein level. Protein levels of the control (24 h) group were set as 1. Values represent the means ± SD (n=3). *P<0.01, compared to the 24 h control group; **P<0.01, compared to the 48 h control group.

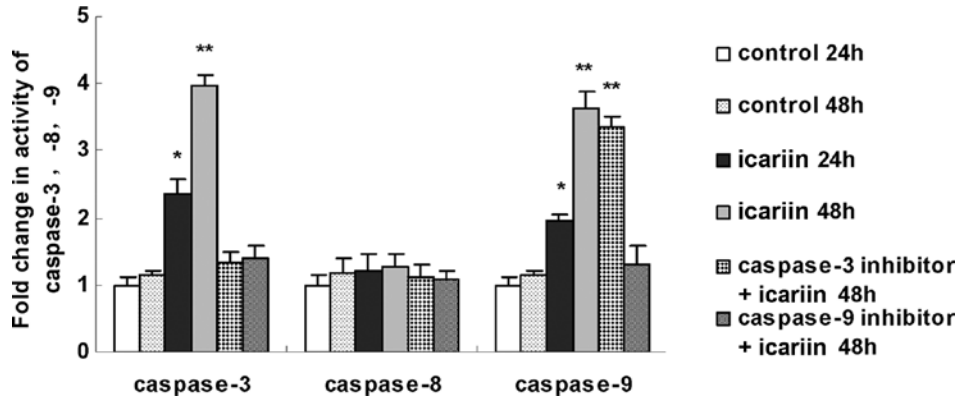

Figure 5. Icariin and caspase activity. (A) Incubation of MLTC-1 with 50 μg/ml icariin for 24 and 48 h activated caspase-9 and -3, but not caspase-8. Pretreatment of MLTC-1 cells with the caspase-3 inhibitor DEVD-CHO (25 μmol/l) only inhibited icariin-induced activation of caspase-3, while, pretreatment of caspase-9 inhibitor z-LEHD-fmk (25 μmol/l) inhibited both caspase-3 and -9 activity. Values are means ± SD (n=3). *P<0.01, compared to 24 h control group; **P<0.01, compared to 48 h control group.

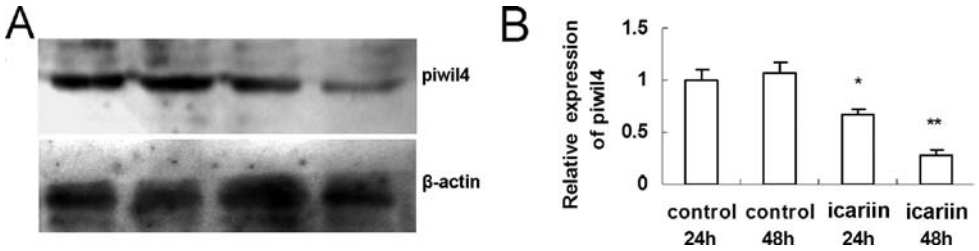

Figure 6. Icariin (50 μg/ml) induces a decrease in piwil4 expression. MLTC-1 cells were treated with 50 μg/ml icariin for 12 and 24 h, then the cells were harvested for Western blot analysis. (A) Representative Western blot analysis for piwil4. (B) Relative expression of piwil4 protein in different groups by normalizing to β-actin. Piwil4 protein levels at control (24 h) group were set as 1. Values represent the means ± SD (n=3). *P<0.05, compared to control (24 h); **P<0.01, compared to control (48 h).

the control group, the percentage of cells in the S phase was slightly decreased by 50 μg/ml icariin after 24 (13.04±0.89% vs. 16.68±1.75%, P<0.05) or 48 h (11.57±0.81% vs. 17.16±3.26%, P<0.05) of treatment.

Icariin induces an alteration in Bcl-2, Bax and cytochrome c expression in MLTC-1 cells. Because of the essential role of the Bcl-2 and Bax proteins in the regulation of apoptosis, we examined the effects of icariin on the expression of these regulatory factors. As shown in Fig. 4, 50 μg/ml icariin treatment decreased the expression of the apoptosis inhibitory protein Bcl-2, while it increased the expression of the pro-apoptotic protein Bax. Also, 50 μg/ml icariin treatment, especially at 48 h, induced a significant increase in the release of the mitochondrial activator of apoptosis, cytochrome c.

Icariin induces differential caspases activation related to the apoptosis in MLTC-1 cells. The activity of caspases-3, -8 and -9 in the different groups was detected to investigate whether caspase proteins were activated by icariin in MLTC-1 cells. As shown in Fig. 5A, activation of both caspase-9 and caspase-3, but not of caspase-8 were observed among icariin treatments at each time-point. Furthermore, compared with icariin alone, pretreatment of MLTC-1 cells with the caspase-3 inhibitor DEVD-CHO (25 μmol/l) inhibited icariin-induced activation of caspase-3, but not of caspase-9, while pretreatment of MLTC-1 with the caspase-9 inhibitor z-LEHD-fmk (25 μmol/l) inhibited icariin-induced activation of both caspase-9 and caspase-3 (both P<0.01).

Piwil4 is involved in icariin-induced apoptosis in MLTC-1 cells. The relative expression of piwil4 protein decreased from 68.3±7.5% (24 h control) and 73±7% (48 h control) to 46±4% (24-h treatment of icariin) and 19±3% (48-h treatment of icariin), respectively, suggesting that piwil4 may be involved in icariin-induced apoptosis in MLTC-1 cells (Fig. 6).

To further investigate whether piwil4 is related to icariin-induced apoptosis, the piwil4-pcDNA™ 6.2-GW/EmGFP miR (piwil4 miR RNAi) vector was constructed to down-regulate the expression of piwil4 in MLTC-1 cells. Compared to the negative control vector, piwil4 miR RNAi vector reduced piwil4 protein levels to 23±4.5% (Fig. 7), suggesting that piwil4 miR RNAi vector effectively inhibited piwil4 expression in MLTC-1 cells.

Subsequently, the effect of piwil4 silencing on the 50 μg/ml icariin-induced alterations of cell viability and cell apoptosis were assessed by MTT and flow cytometry analysis, respectively. Compared to parental MLTC-1 cells or MLTC-NC cells, piwil4 silencing exhibited no direct effect on cell viability (P>0.05). However, on the condition of icariin stimulation, a decrease in cell viability was observed (P<0.05). Similarly, piwil4 silencing showed no effect on cell apoptosis compared

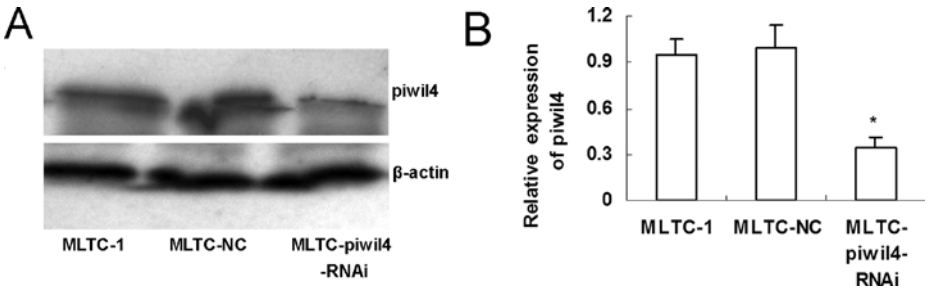

Figure 7. The Piwil4 miR RNAi vector reduces piwil4 protein levels. MLTC-1, parental MLTC-1 cells; MLTC-NC, MLTC-1 cells transduced with negative control miR RNAi vector; MLTC-piwil4-RNAi, MLTC-1 cells transduced with piwil4 miR RNAi vector. (A) Representative Western blotting of piwil4 in MLTC-1 cells of different groups. (B) Relative expression of piwil4 protein in different groups by normalizing to β-actin protein. Piwil4 protein levels at MLTC-NC control group were set as 1. Values represent the means ± SD (n=3). *P<0.01, compared to MLTC-NC group or MLTC-1 group.

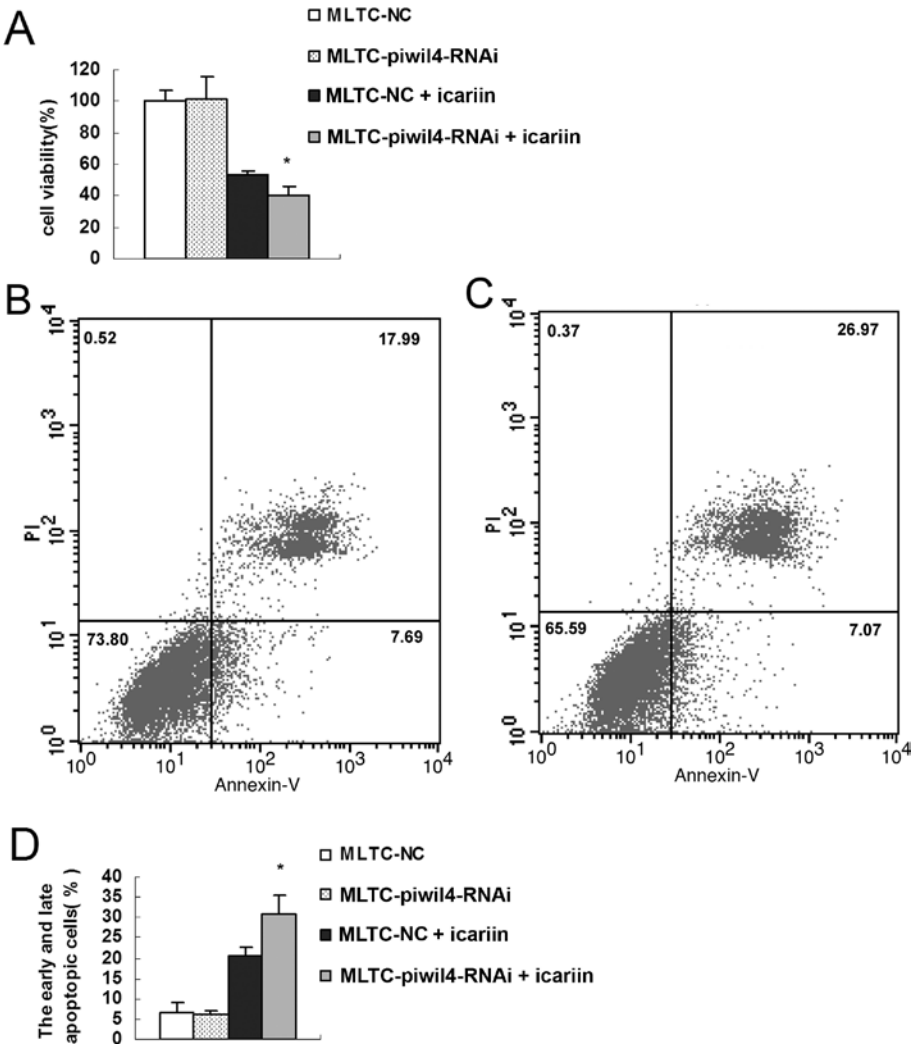

Figure 8. Piwil4 silencing enhances the effect of icariin on MLTC-1 cells.MLTC-NC (transfected with negative miR RNAi vector) and MLTC-1-piwil4-RNAi cells (transfected with piwil4 miR RNAi vector) were treated with 50 μg/ml icariin for 48 h. The MTT and flow cytometry assay were then performed to investigate the cell viability and cell apoptosis, respectively. The results are from three experiments and are presented as means ± SD. (A) Cell viability was assessed by the MTT assay. (B) and (C) Representative histograms of cell apoptosis analyzed by flow cytometric in icariin-treated MLTC-1-NC and MLTC-1-piwil4-RNAi cells. (D) The percentage of cell apoptosis for each treatment group. All the values represent the means ± SD (n=3). *P<0.01, compared to the icariin-treated MLTC-NC group.

to parental MLTC-1 cells or MLTC-NC cells (data not shown), but enhanced the pro-apoptosis effect induced by icariin (Fig. 8B and C). These results indicate that piwil4 plays a role in the icariin-induced cytotoxicity in MLTC-1 cells.

Discussion

Several studies have been performed to explore new treatments for Leydig tumors, such as imatinib and cordycepin. Imatinib,

a tyrosine kinase inhibitor, has activity in gastrointestinal stromal tumors and in a variety of other malignant tumors (18,19). Studies *in vitro* and in a mouse model have suggested that imatinib may also be active in malignant Leydig cell tumors (20). Cordycepin a constituent from the mycelia of *Cordyceps sinensis* (CS), is considered an active component with antitumor effects in mouse melanoma and lung carcinoma cells and in human oral cancer cells (21,22). In addition, Jen *et al* have demonstrated that cordycepin exhibited an antitumor effect in Leydig cell tumors (23).

In this study, we showed that icariin could inhibit the growth of MLTC-1 Leydig tumor cells *in vitro*. A cell proliferation assay indicated that icariin in a concentration range from 25 to 100 μg/ml had time- and dose-dependent growth inhibitory effects on MLTC-1 cells. The decrease in cell proliferation may be due to an increase in cell apoptosis. In line with this expectation, compared to controls, the percentage of early- and late-apoptotic cells increased to 17.1±1.5% and 24.7±1.6% by exposure to 50 μg/ml icariin for 24 and 48 h, respectively.

Previous studies have shown an association between cell progression and cancer, and targeting the cell cycle has become an appreciated strategy for tumor treatment (24,25). The cell cycle profile was analyzed in MLTC-1 cells with icariin treatment. The percentage of cells in the S phase was slightly decreased with 50 μg/ml icariin stimulation for 24 or 48 h, while alterations of the G_0/G_1 and G_2/M cell populations showed no significant differences between the icariin-treated and the control groups. These results demonstrate that icariin causes an alteration in cell cycle progression of MLTC-1 cells. Collectively considering the results from cell apoptosis and cell cycle assays, it was speculated that the inhibition of tumor cell proliferation by icariin was mainly due to an increase in cell apoptosis.

The proteins of the Bcl-2 family, including Bcl-2 and Bax, are key regulators of apoptosis and may act as balancing factors for the cell apoptotic program (26). Bcl-2 related proteins are primarily localized in the outer mitochondrial, nuclear membranes and the endoplasmic reticulum (27). Bcl-2 may function as a protective factor against apoptosis triggered by cytochrome c release from the mitochondria. Bax, a pro-apoptotic protein, is located in the cytosol or loosely attached to membranes. In response to apoptotic stimulus, Bax translocates form the cytosol to the mitochondria, resulting in cytochrome c release and caspases activation (28,29). The Bcl-2 and Bax proteins are sensitive to apoptotic agents, and the ratio of Bcl-2 and Bax is considered to be a good predictive marker for cell will survival or death (30). Here, results of Western blotting indicated that icariin lowered the Bcl-2/Bax protein expression ratio, implying that the antitumor effects of icariin are elicited through regulation of the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. In addition, accompanying the Bax expression increase, cytochrome c was elevated by icariin stimulation, which further confirms the role of Bcl-2/Bax expression in the icariin-induced MLTC-1 cell apoptosis.

Caspases, the important members of the apoptotic machinery, are a large family of proteases, including the upstream initiators (caspase-2, -8, -9, -10, -12) and the downstream effectors (caspase-3, -6, -7) (28). Among these caspases, caspase-8 is activated in death receptor-mediated apoptosis, while caspase-9, as the upstream caspase in the cytochrome c pathway for apoptosis, is triggered by the release of cytochrome c from mitochondria (31).

In order to examine the important roles of caspase-8 and -9 in apoptosis as described above, we next evaluated whether caspase-8, -9 as well as the downstream effector, caspase-3 are involved in the apoptotic effects of icariin. It is evident that icariin treatment resulted in activation of caspase-9 and -3, other than caspase-8. To further confirm the change of caspase-9 and -3 in the icariin-treated MLTC-1 cells, the caspase-9 or -3 inhibitor was added to the media. The results demonstrate that the caspase-3 inhibitor only inhibited icariin-induced activation of caspase-3, while the caspase-9 inhibitor inhibited both caspase-3 and -9 activity, which indicated that the effector caspase-3 is triggered by the upstream molecule, caspase-9. These results suggest that icariin can induce apoptosis by activating caspase-9, and the downstream effector, caspase-3.

Piwil4 belongs to the Ago/Piwi family, which is composed of two conserved domains, PAZ and PIWI. The Ago/PIWI proteins have been shown to interact with the ribonuclease (RNase) III enzyme Dicer that catalyzes the maturation of miRNA and siRNA. Within the family, four members of the PIWI-like family, PIWIL1, PIWIL2, PIWIL3 and PIWIL4, have been identified in humans in previous reports, while in the mouse, there are only three Piwi subfamily genes, piwil1, piwll2 and piwil4 (32-35). In regards to piwil4 and its tissue distribution, research has mainly focused on its role in spermatogensis (36-38).

We find that piwil4 is expressed in MLTC-1 cells, and interestingly, MLTC-1 cell apoptosis induced by icariin is related to piwil4. Western blotting results illustrate that the relative expression of piwil4 protein was decreased by treatment with 50 μg/ml icariin in a time-dependent manner. To examine whether piwil4 is involved in icariin-induced apoptosis, we used miR RNAi vector to down-regulate the expression of piwil4 in MLTC-1 cells. MTT and flow cytometry results demonstrate that piwil4 silencing enlarged the effect of icariin on cell viability and cell apoptosis, which indicated that piwil4 may be an anti-apoptotic factor, while icariin could exert its antitumor activity by down-regulating piwil4 expression.

In summary, we have shown that icariin has pro-apoptotic activity in MLTC-1 cells by regulating the expression of Bcl-2/Bax, the release of cytochrome c and the activity of caspase-9 and -3. Additionally, piwil4, an important regulator of spermatogenesis, is involved in the icariin-induced cell apoptosis. In reference to its antitumor activity, icariin may be a therapeutic candidate on testicular Leydig cell tumors.

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