

Original Article

2-Methoxyestradiol improves the apoptosis level in keloid fibroblasts through caspase-dependent mechanisms *in vitro*

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Abstract: Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Fibroblasts are the main cellular ingredients in keloid tissue, which has a relatively low apoptosis level. A natural metabolite of estradiol, 2-Methoxyestradiol (2ME2) exerts a pro-apoptotic effect on tumor cells. In this study, the expression levels of key factors in the apoptosis pathway and the expression level of the proliferating cell nuclear antigen (PCNA) were measured to assess the levels of apoptosis and proliferation in both normal skin fibroblasts and keloid fibroblasts. Twelve samples were obtained from 12 patients: 6 keloid patients and 6 non-keloid patients. All 12 of the patients were randomly selected from the Department of Plastic Surgery at Peking Union Medical College Hospital from June 2016 to December 2016. After cell culture, fibroblasts were divided into the following 6 groups: normal skin fibroblasts (S); keloid fibroblasts (K); keloid fibroblasts treated with 2ME2 (2ME2); keloid fibroblasts treated with DMSO (DMSO); keloid fibroblasts treated with the caspase inhibitor Ac-DEVD-CHO (IN); and keloid fibroblasts treated with both Ac-DEVD-CHO and 2ME2 (IN+2ME2). Fibroblasts at up to passage 3 were used for analysis. Cell activity was measured by the cell counting kit-8. TUNEL staining was used to observe the cell apoptotic morphology. The key apoptosis factors (caspase-3, caspase-8, caspase-9, Bcl-2, Bax, and cytochrome-c) and PCNA expression levels were detected by immunofluorescence analysis and Western blotting. A certain concentration of 2ME2 was also used in group S to evaluate the toxicity. Compared with that in the other groups, 2ME2 significantly inhibited cell activity and led to apoptotic appearance of fibroblasts. In protein analysis, 2ME2 remarkably increased the expression of apoptosis factors and decreased the PCNA expression. Apoptosis levels were reduced by both the caspase inhibitor and 2ME2; thus indicating that the pro-apoptosis effect of 2ME2 was achieved through a caspase-dependent mechanism in keloid fibroblasts. Toxicity assessment showed that 2ME2 had a very low influence on normal skin fibroblasts. 2ME2, considered to be a new promising type of chemotherapy drug, exerts a pro-apoptosis effect by regulating the caspase family and an anti-proliferation effect towards keloid fibroblasts, and it presents low toxicity towards normal fibroblasts *in vitro*.

Keywords: Keloid, fibroblast, 2-Methoxyestradiol, apoptosis, caspase, proliferation

Introduction

There are three major types of morphologically distinct cell death that occur in multicellular organisms: apoptosis, autophagic cell death, and necrosis. Apoptosis, also defined as programmed cell death, is triggered when cell-surface death receptors are bound with ligands or when Bcl-2 family pro-apoptotic proteins cause

the permeabilization of the mitochondrial membrane [1]. In this process, caspase-8 receives the cell apoptosis signal from death receptors and assembles Bax on the mitochondrial membrane, which changes the Bcl-2/Bax ratio and releases cytochrome-c (cyt-c) from mitochondria into the cytoplasm. Then, the activated caspase-9 and caspase-3 further manage the apoptosis process [2, 3].

2ME2 improves keloid fibroblast apoptosis through the caspase family

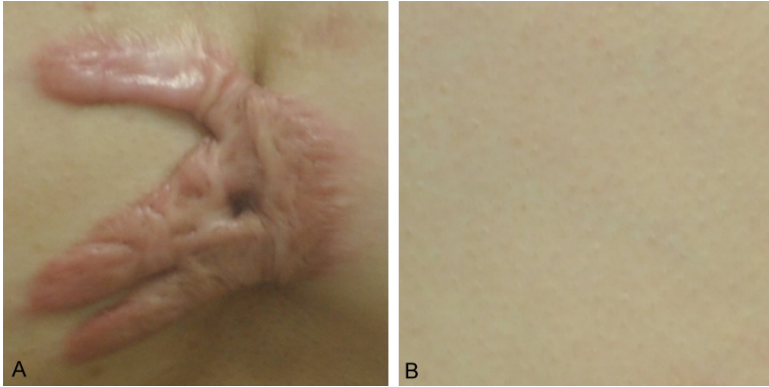


Figure 1. Sampling conditions in two types of patients: (A) keloid tissue from keloid patients; and (B) normal skin tissue from non-keloid patients.

A naturally occurring derivative of estradiol, 2-Methoxyestradiol (2ME2) has been shown to be an orally active, well-tolerated small molecule that possesses anti-tumor activity [4]. It has been regarded as a new promising chemotherapy drug that can inhibit cell growth and induce cell death in a variety of malignant cell lines [5]. The mechanisms of action of 2ME2 include the induction of apoptosis, the inhibition of cell proliferation and angiogenesis, and an increase in radiation sensitivity [6-10].

A keloid, regarded as the end product of abnormal wound healing, is defined as excessive scar tissue formation extending beyond the area of the original skin injury and occurring in predisposed individuals [11]. Pain, itching, functional limitation, and disfigurement often cause psychological distress and seriously affect keloid patients' quality of life [12]. In our previous study, we observed that keloid tissue has a similar apoptosis level as physiological scars and normal skin but it has a higher expression of PCNA, indicating that keloid scars have high levels of proliferation and normal apoptosis [13]. In this case, the inhibition of proliferation and promotion of apoptosis is the key approach to keloid non-surgical treatment. In 1996, Appleton [14] first reported the degree of apoptosis and proliferation in keloid tissue, and most of the studies have focused on apoptosis in keloid fibroblasts, which are the main cellular ingredients of keloid tissue. However, most of the studies have only concentrated on the apoptosis phenomenon that is caused by certain drugs instead of exploring the drug mechanism of action or toxicity. In this study, we focused on the effect and mechanism of action of 2ME2 towards keloid fibroblasts

by measuring the expression of key factors in the apoptosis pathway (caspase-3, caspase-8, caspase-9, Bcl-2, Bax, and Cyt-c) and the expression of proliferating cell nuclear antigen (PCNA). In addition, we also determined the toxicity of 2ME2 in normal skin fibroblasts to evaluate its safety.

Materials and methods

Patients, samples, cell culture, and treatment

The study protocol was reviewed and approved by the Bioethical Committee of Peking Union Medical College Hospital. All of the patients gave informed consent. Twelve patients (age ranging from 18 to 50 years) were randomly selected from the Department of Plastic Surgery at Peking Union Medical College Hospital from June 2016 to December 2016: 6 keloid patients (3 males and 3 females, median age: 31 years) and 6 non-keloid patients (3 males and 3 females, median age: 32 years) (**Figure 1**). All of the samples were obtained from the chest region. Keloid samples were taken from the core of the keloid tissues. No significant difference in age, sex, or site was observed between the keloid patients and the non-keloid patients ($P > 0.05$). Keloids were caused by trauma, and they were diagnosed by pathological examination. None of the patients reported history of any systemic disorders, history of taking drugs, or history of receiving other treatments that might influence the study results.

After obtaining the keloid core, the samples were minced into 0.2-0.3 cm³ blocks and laid onto 75 cm² culture flasks. After 4 h of adherence, 5-7 ml of Dulbecco's minimal essential medium (Gibco, Big Cabin, OK, USA) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U mL⁻¹), and streptomycin (100 µg mL⁻¹) (Gibco) were poured into the flasks. Then, the flasks were maintained at 37°C in a 5% carbon dioxide-enriched humidified atmosphere. The culture medium was changed every 3 days [10]. The normal skin fibroblast cell culture was performed based on the study by Vangipuram [15]. Fibroblasts at up to passage 3 were used in this study.

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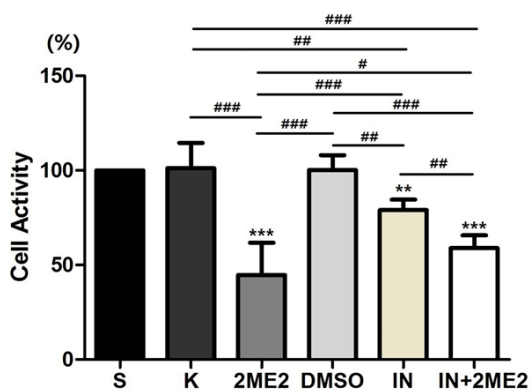


Figure 2. Cell activity in all groups at 48 h after treatment. The values are expressed as mean \pm SD ($n = 6$ in each group; ** $P < 0.01$, *** $P < 0.001$ vs. group S; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$).

Fibroblasts were divided into the following 6 groups: 1. Group S: normal skin fibroblasts from normal skin tissue; 2. Group K: keloid fibroblasts from keloid tissue; 3. Group 2ME2: keloid fibroblasts from keloid tissue treated with 6.975 μM (IC_{50} from our pre-experiment) 2ME2 (10 mM in 1 ml DMSO, Selleck Chemicals, Houston, Texas, USA); 4. Group DMSO: keloid fibroblasts from keloid tissue treated with 0.7% DMSO (based on 2ME2 solvent); 5. Group IN: keloid fibroblasts from keloid tissue treated with 8 μM caspase inhibitor Ac-DEVD-CHO (Selleck Chemicals); 6. Group IN+2ME2: keloid fibroblasts from keloid tissue treated with both 8 μM caspase inhibitor Ac-DEVD-CHO and 6.975 μM 2ME2.

Cell activity assessment

Cell activity was detected by using the CCK-8 assay (Dojindo, Kumamoto, Japan). Fibroblasts were seeded at 5000 cells/well in 96-well plates according to the above-mentioned grouping for 48 h. The protocols were based on the kit instructions. The optical density was measured at 450 nm by a microplate reader.

TdT-mediated dUTP-X nick end labeling (TUNEL) staining

TUNEL staining was used to observe programmed cell death, which was assessed with the In Situ Cell Death Detection Kit (POD, Roche, Basel, Switzerland). Fibroblasts were seeded at 5000 cells/well in 96-well plates according to the above-mentioned grouping for 24 h. All of the staining procedures were based on the kit instructions.

Immunofluorescence analysis

Fibroblasts were seeded at 10000 cells/well in 24-well plates according to the above-mentioned grouping for 24 h. Each well was fixed with 4% paraformaldehyde for 30 min, followed by washing 2 times with phosphate buffered saline (PBS). Then, each well was incubated with 0.3% Triton-100 for 15 min. Goat serum was used for blocking for about 60 min. After that, each well was incubated with anti-caspase-3 (1:200, Abcam, Cambridge, UK), anti-caspase-8 (1:200, Abcam), anti-caspase-9 (1:200, Abcam), Bcl-2 (1:200, Abcam), Bax (1:200, Abcam), cyt-c (1:200, Abcam), and PCNA (1:200, Abcam) antibodies in a humidified chamber at 4°C overnight (12-16 h). Primary antibody was labeled by anti-rabbit IgG H&L (Dylight 488) (1:200, Abcam) for 1 h in the dark area. After washing 3 times with PBS, each well was stained with 10 mg/ml Hoechst 33258 (Sigma-Aldrich, USA) for 20 min. A Zeiss Axiophot fluorescence microscope (Axio-Cam MRC, Zeiss, Oberkochen, Germany) and Axio-vision Zeiss software were used to observe the expression of factors. Green areas represent the positive areas in a cell. The shade of the green area represents the expression level. Blue areas represent the area of the DNA.

Western blot detection

Cell Total Protein Extraction Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to extract protein from fibroblasts. After 24 h of treatment, cells were collected and incubated on ice for 10 min in a cell lysis buffer (246 μl of lysis buffer, 1.25 μl of phosphatase inhibitor, 0.25 μl of protease inhibitor, and 2.5 μl of PMSF) and centrifuged for 15 min (4°C, 14000 rpm). Equal amounts of supernatant protein (30 μg) were separated on a 10% SDS-PAGE gel. Then, protein was transferred onto nitrocellulose membranes for immunoblotting. A blocking buffer (Li-cor, Lincoln, USA) was used to block the Western blot membrane for 2 h. After that, the membrane was incubated with anti-caspase-3 (1:500, Abcam), anti-caspase-8 (1:200, Abcam), anti-caspase-9 (1:200, Abcam), Bcl-2 (1:200, Abcam), Bax (1:200, Abcam), cyt-c (1:200, Abcam), and PCNA (1:200, Abcam) antibodies in a humidified chamber at 4°C overnight for 12-16 h. The membranes were incubated with secondary antibodies (Li-cor, Lincoln, NB) at 1:10000 dilution for 1 h in the dark. A double-color infra-

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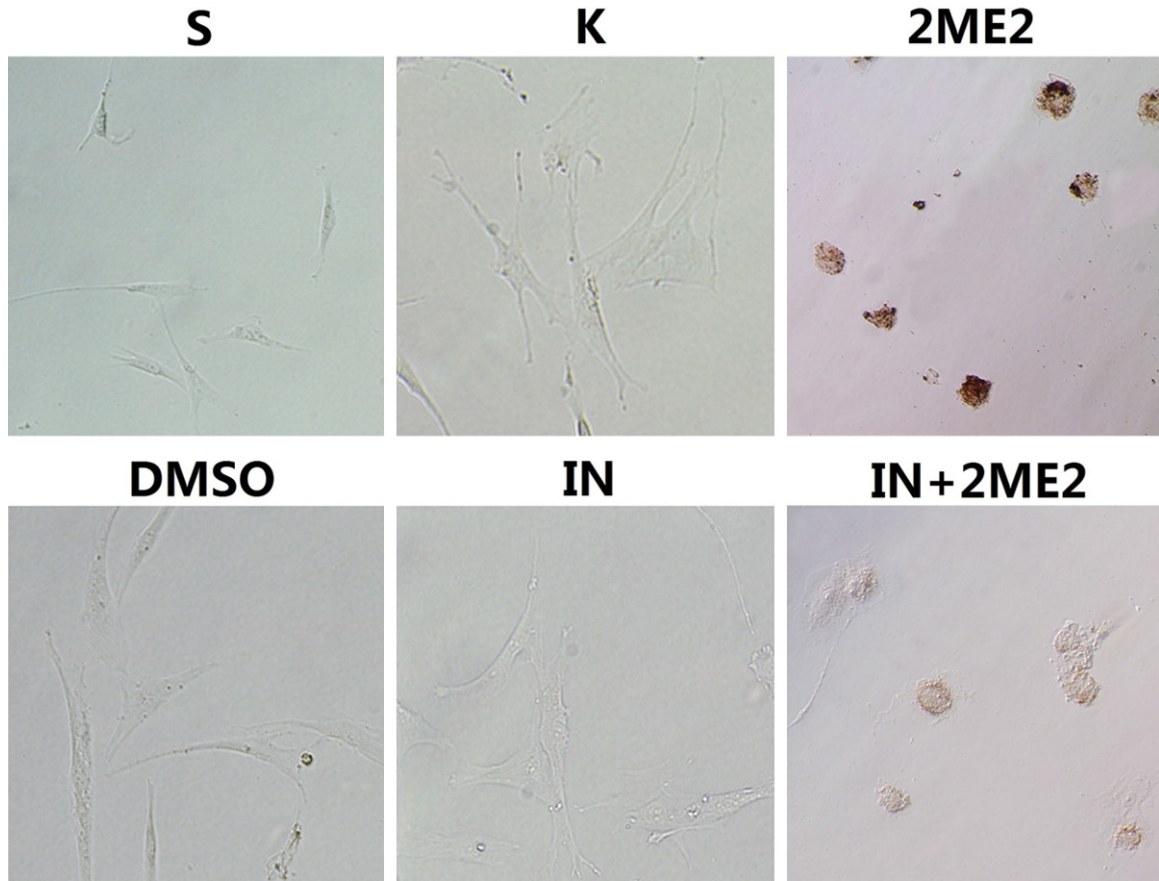


Figure 3. TUNEL staining in all groups 24 h after treatment. Fibroblasts in groups S, K, DMSO, and IN showed basically normal morphology with a clear nucleus and cytoplasm. However, fibroblasts in groups 2ME2 and IN+2ME2 showed a typical apoptotic appearance, such as cell shrinkage, chromatin pyknosis, and karyorrhexis. Compared with group 2ME2, the extent of apoptotic fibroblasts was much less in group IN+2ME2.

red laser imaging system (Odyssey, Li-cor) was used for density detection.

2ME2 toxicity evaluation

In order to evaluate the toxicity of 2ME2, the fibroblasts were divided into the following two groups: fibroblasts from normal skin (S) and fibroblasts from keloid tissue (K). All of the groups were treated with 6.975 μ M 2ME2. Fibroblasts were seeded at 5000 cells/well in 96-well plates according to the above-mentioned grouping for 6 h, 12 h, 24 h, and 48 h. CCK-8 assay was used to assess the difference in cell activity between normal skin fibroblasts and keloid fibroblasts at different time points.

Statistical analysis

Study data are presented as means \pm standard deviation (means \pm SD). SPSS Statistics 24.0

software (SPSS, Inc., Chicago, IL) was used for statistical analysis. The independent sample t test was used for 2ME2 toxicity evaluation. One-way analysis of variance (ANOVA) followed by the LSD t test were used for other statistical analysis. Statistical significance was set at $P < 0.05$.

Results

2ME2 significantly decreased the keloid fibroblast activity

Compared with group S (considered as 1) and group K (1.01 ± 0.13), fibroblast activity in group 2ME2 (0.45 ± 0.17) was remarkably decreased (2ME2 vs. S, $P < 0.001$; 2ME2 vs. K, $P < 0.001$). There was no significant difference between group DMSO (1.00 ± 0.08) and group K. Compared with group 2ME2, the cell activity

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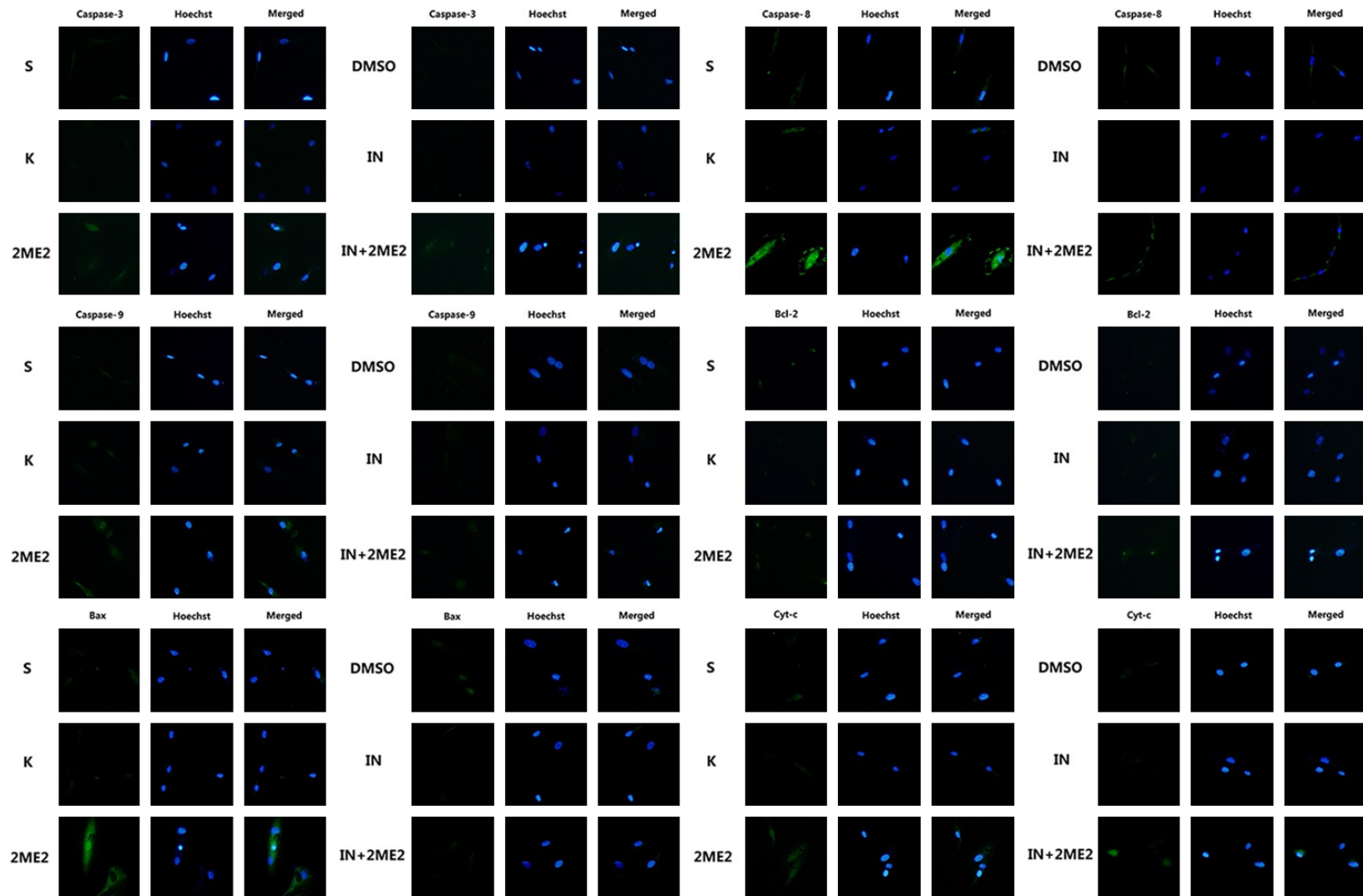


Figure 4. Images (400×) of immunofluorescence staining of all of the factors. The green area represents cells with positive expression of the target protein, whereas the blue area represents the area of the DNA. The expression levels of all of the factors were much higher in group 2ME2. Compared with group 2ME2, levels of the key factors in group IN+2ME2 were decreased due to the effect of the caspase inhibitor (n = 6 in each group).

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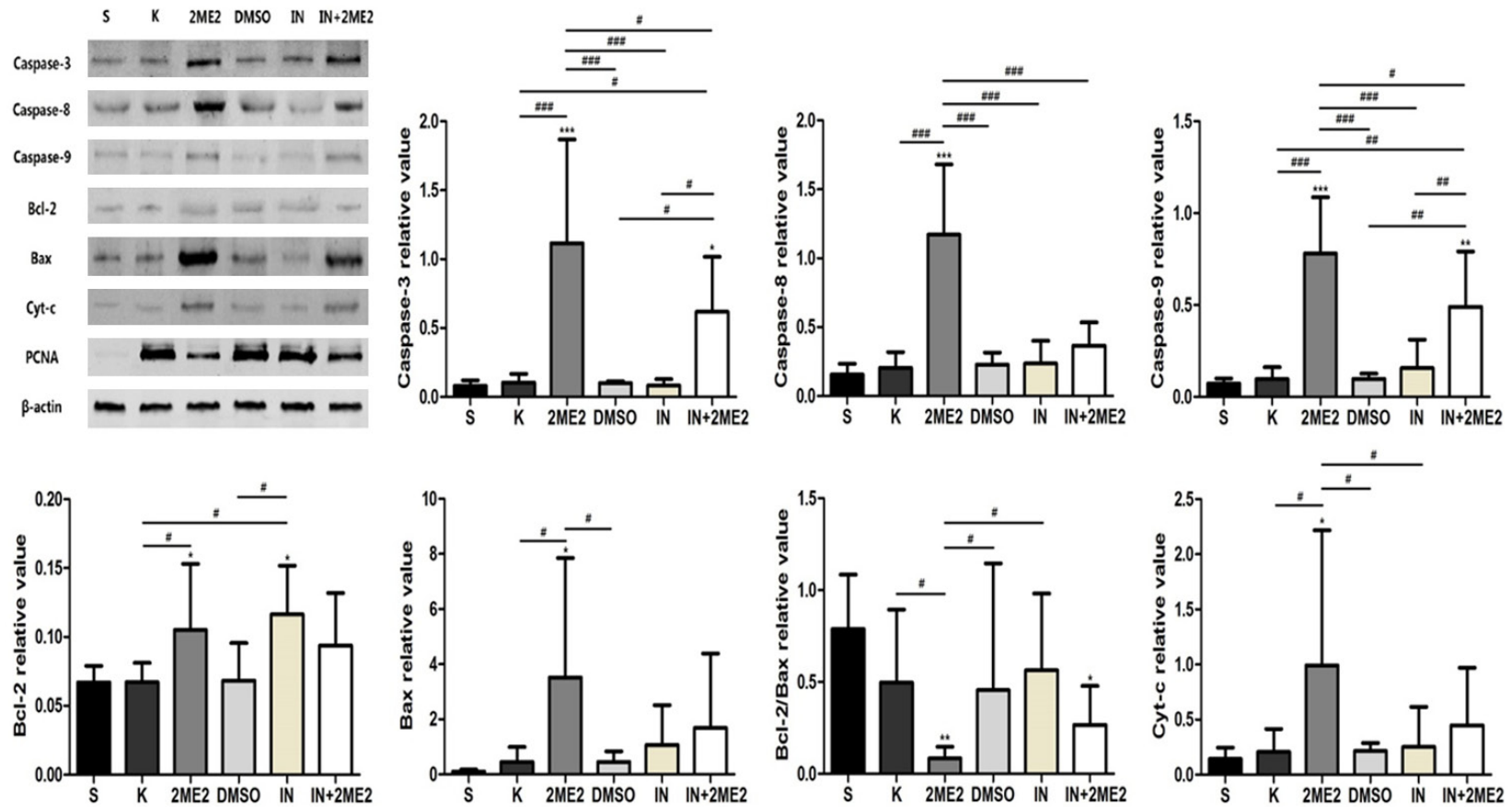


Figure 5. Relative protein amounts for all target proteins. Similar to the immunofluorescence results, the expression levels of caspase-3, caspase-8, caspase-9, Bcl-2, Bax, and cyt-c were significantly increased by 2ME2 treatment. Compared with groups S and K, group DMSO showed no significant difference in the expression of key factor. Although 2ME2 increased Bcl-2 and Bax expression, the Bcl-2/Bax ratio was remarkably decreased. Compared with group 2ME2, expression of key factors was decreased in group IN+2ME2. Representative images of Western blots of all factors are shown in the upper left corner. Values are expressed as means \pm SD (n = 6 in each group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. group S; #P < 0.05, ###P < 0.01, ####P < 0.001).

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Table 1. Protein relative value of all markers in each group. Values are means \pm SD (n = 6 in each group)

Factor	Protein relative value						P value		
	S	K	2ME2	DMSO	IN	IN+2ME2	< 0.05	< 0.01	< 0.001
Caspase-3	0.08 \pm 0.04	0.10 \pm 0.06	1.12 \pm 0.75	0.10 \pm 0.01	0.08 \pm 0.05	0.62 \pm 0.40	S vs. IN+2ME2 K vs. IN+2ME2 2ME2 vs. IN+2ME2 DMSO vs. IN+2ME2 IN vs. IN+2ME2		S vs. 2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN
Caspase-8	0.16 \pm 0.08	0.20 \pm 0.12	1.17 \pm 0.51	0.23 \pm 0.09	0.24 \pm 0.16	0.37 \pm 0.17			S vs. 2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN 2ME2 vs. IN+2ME2
Caspase-9	0.07 \pm 0.03	0.10 \pm 0.06	0.78 \pm 0.31	0.10 \pm 0.03	0.16 \pm 0.15	0.49 \pm 0.30	2ME2 vs. IN+2ME2	S vs. IN+2ME2 K vs. IN+2ME2 DMSO vs. IN+2ME2 IN vs. IN+2ME2	S vs. 2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN
Bcl-2	0.07 \pm 0.01	0.07 \pm 0.01	0.11 \pm 0.05	0.07 \pm 0.03	0.12 \pm 0.04	0.09 \pm 0.04	S vs. 2ME2 S vs. IN K vs. 2ME2 K vs. IN DMSO vs. IN		
Bax	0.11 \pm 0.07	0.45 \pm 0.54	3.52 \pm 4.33	0.46 \pm 0.38	1.07 \pm 1.44	1.68 \pm 2.70	S vs. 2ME2 K vs. 2ME2 2ME2 vs. DMSO		
Bcl-2/Bax	0.79 \pm 0.30	0.50 \pm 0.40	0.08 \pm 0.06	0.46 \pm 0.69	0.56 \pm 0.42	0.27 \pm 0.21	S vs. IN+2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN	S vs. 2ME2	
Cyt-c	0.14 \pm 0.10	0.21 \pm 0.21	0.99 \pm 1.23	0.21 \pm 0.07	0.25 \pm 0.36	0.45 \pm 0.52	S vs. 2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN		
PCNA	0.27 \pm 0.04	2.17 \pm 1.02	0.82 \pm 0.15	2.29 \pm 0.50	1.95 \pm 1.01	1.41 \pm 0.70	DMSO vs. IN+2ME2	S vs. IN+2ME2 K vs. 2ME2 2ME2 vs. DMSO 2ME2 vs. IN	S vs. K S vs. DMSO S vs. IN

Protein relative value in all groups.

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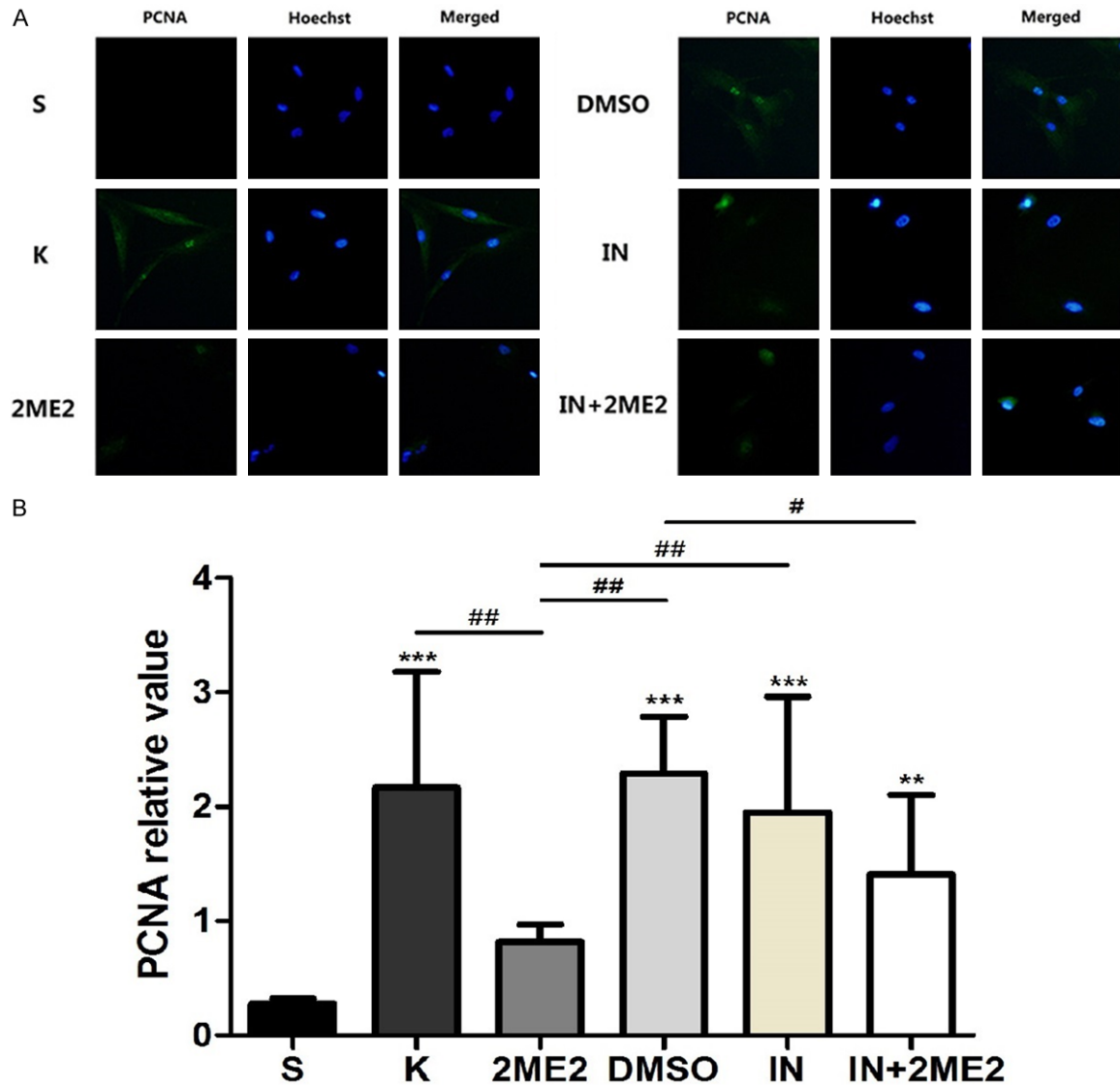


Figure 6. Immunofluorescence staining and western blot data analysis of PCNA expression in all groups. A. Immunofluorescence staining images. PCNA showed similar high expression in groups K, DMSO, and IN. 2ME2 significantly decreased the PCNA expression in keloid fibroblasts. B. PCNA relative value. Compared with groups K, DMSO, and IN, the PCNA expression was remarkably reduced with 2ME2 treatment. Values are expressed as means \pm SD (n = 6 in each group; ** $P < 0.01$, *** $P < 0.001$ vs. group S; # $P < 0.05$, ## $P < 0.01$).

was increased in group IN+2ME2 (0.59 ± 0.07) ($P < 0.05$) (Figure 2).

2ME2 transforms keloid fibroblasts into apoptotic phenotype

Figure 3 shows the TUNEL staining results in different groups. In groups S, K, DMSO, and IN, fibroblasts showed basically normal morphology, which appears as a long spindle-shaped form with clear nuclei and cytoplasm. Fibroblasts in group 2ME2 showed apoptotic ap-

pearance, such as cell shrinkage and rounding, chromatin pyknosis, and karyorrhexis. Fibroblasts in group IN+2ME2 also showed apoptotic appearance, but to a lesser extent.

2ME2 increased the expression of key factors in the apoptosis pathway

Expression analysis of caspase-3, caspase-8, caspase-9, Bcl-2, Bax, and cyt-c was performed by immunofluorescence and Western blot studies. In immunofluorescence images, group

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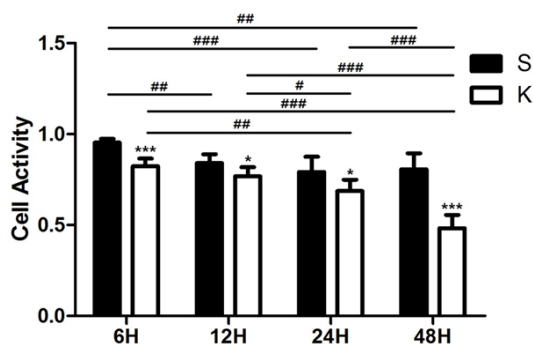


Figure 7. Cell activity in groups S and K (all treated with 6.975 μ M 2ME2) at different time points. The values are expressed as mean \pm SD (n = 6 in each group; * P < 0.05, *** P < 0.001 vs. group S; # P < 0.05, ## P < 0.01, ### P < 0.001).

2ME2 showed the highest expression level of key factors in the apoptosis pathway. There were no obvious changes in the expression of key factors among groups S, K, DMSO, and IN (Figure 4).

According to Western blot results, 2ME2 significantly increased the expression of key factors in the apoptosis pathway, such as caspase-3, caspase-8, caspase-9, and cyt-c, compared to that in the other groups. No significant differences were found in the expression of caspase-3, caspase-8, caspase-9, and cyt-c among groups IN, S, and K. However, the caspase inhibitor increased the Bcl-2/Bax ratio and reduced the cell apoptosis level. Although the expression levels of Bcl-2 and Bax were also increased by 2ME2 treatment, the Bcl-2/Bax ratio was remarkably decreased. Compared with group 2ME2, levels of the above-mentioned key factors, especially caspase-3, caspase-8, and caspase-9, in group IN+2ME2 were decreased due to the effect of the caspase inhibitor (Figure 5; Table 1).

2ME2 reduced the PCNA expression in keloid fibroblasts

The PCNA expression level was evaluated by immunofluorescence and Western blot. In all of the keloid groups, fibroblasts showed higher PCNA expression that was remarkably decreased by 2ME2 treatment (Figure 6A). Qualitative analysis by western blot showed similar results. No significant difference in PCNA expression was observed between groups K and DMSO, indicating that 0.7%

DMSO has no effect on keloid fibroblast proliferation. The anti-proliferation effect of 2ME2 was also decreased by the caspase inhibitor in group IN+2ME2 (Figure 6B; Table 1).

2ME2 showed low toxicity towards normal skin fibroblasts

CCK-8 was used to evaluate the 2ME2 toxicity towards normal skin fibroblasts. In this study, both normal fibroblasts and keloid fibroblasts were treated with 6.975 μ M 2ME2 for 6 h, 12 h, 24 h, and 48 h. In group S, significant differences were observed between the 6 h and other time point groups, implying that 2ME2 could affect the normal fibroblast cell activity from 6 h to 12 h, and, after using 2ME2 for more than 12 h, the normal fibroblast cell activity remained at a high level and was slightly changed. On the contrary, with passage of time, the keloid fibroblast cell activity started to decrease. At each time point, there was a significant difference between groups S and K (12 h & 24 h, P < 0.05; 6 h & 48 h, P < 0.001) (Figure 7; Table 2).

Discussion

Apoptosis, also known as programmed cell death, occurs in multicellular organisms and it leads to characteristic cell changes and death. The changes include blebbing, cell shrinkage, nuclear fragmentation, chromosomal DNA fragmentation, and chromatin condensation [16]. Many signaling pathways and key factors play a paramount role in the apoptosis process, which may be activated by inflammatory factors and stress conditions. Caspase-8 expression increases when cell-surface death receptors are bound with ligands, thus magnifying the programmed cell death signal and delivering this signal to the mitochondria [17-19]. Then, Bax is assembled on the mitochondrial membrane and it decreases the Bcl-2/Bax ratio, leading to the release of cyt-c from the mitochondria to the cytoplasm. In the cytoplasm, cyt-c combines with Apaf-1 to become the apoptosome with the dATP/ATP unit, which activates the initiator caspase (caspase-9). Once caspase-3 is activated, it promotes apoptosis as a result of the above-mentioned morphological changes [20-22].

2ME2 is generated by a sequential hydroxylation of estradiol through the enzyme cyto-

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Table 2. Cell activity of different times in S and K groups (all treated with 6.975 μ M 2ME2). Values are means \pm SD (n = 6 in each group)

Time	Cell Activity				P value
	S	S group P value	K	K group P value	
6 h	0.95 \pm 0.02	6 h vs. 12 h, P < 0.01	0.82 \pm 0.04	6 h vs. 24 h, P < 0.01	P < 0.001
12 h	0.84 \pm 0.05	6 h vs. 24 h, P < 0.001	0.77 \pm 0.05	6 h vs. 48 h, P < 0.001	P < 0.05
24 h	0.79 \pm 0.08	6 h vs. 48 h, P < 0.01	0.69 \pm 0.06	12 h vs. 24 h, P < 0.05	P < 0.05
48 h	0.81 \pm 0.09		0.48 \pm 0.07	12 h vs. 48 h, P < 0.001 24 h vs. 48 h, P < 0.001	P < 0.001

Cell activity in all groups.

chrome P450 isoform 1A1 to produce 2-hydroxyestradiol followed by a conjugation reaction catalyzed by the enzyme Catechol-O-Methyltransferase generating 2ME2 from 2-hydroxyestradiol [23]. Presently, it is an investigational drug that is considered to be a potential cancer chemotherapeutic agent, which can inhibit cell growth and induce cell death in a variety of malignant cell lines, including lung and colon carcinomas, melanoma, and reproductive system cancers [23-28]. Its anticancer activity has been attributed to inhibition of angiogenesis [4, 28], induction of apoptosis [29], and inhibition of β -tubulin polymerization [30].

Unlike hypertrophic or physiological scars, keloids tend to outgrow the original boundary of the wound and they do not regress, and this type of keloid has been regarded as a benign dermal tumor [14]. The high recurrence of keloids makes it a refractory disease in plastic surgery. In our previous study, we observed that keloid tissue has a similar apoptosis level as physiological scars and normal skin but it has a higher expression of PCNA [13]. In such a case, the promotion of apoptosis and inhibition of the proliferation of keloid fibroblasts is a key point in keloid treatment. In this study, we assessed whether 2ME2 has a double effect (cell growth inhibition and cell death induction) towards keloid fibroblasts and we verified the mechanism by using a caspase inhibitor.

PCNA is a homotrimer that achieves its activity by acting as a scaffold that encircles the DNA and recruits proteins involved in DNA replication, DNA repair, chromatin remodeling, and epigenetics [31]. PCNA expression is upregulated in proliferating tissue, especially tumors [32, 33], making it an effective indicator of the tissue proliferation level. Research by Massaro demonstrated that 2ME2 has an inhibitory

effect on proliferation and invasion in human melanoma cells [7]. In our study, keloid fibroblast activity and PCNA expression were significantly decreased by 2ME2 treatment. The effect of 2ME2 towards keloid fibroblasts was evaluated by TUNEL staining. In groups S, K, DMSO, and IN, the cells basically maintained their normal appearance with a clear nucleus and cytoplasm. 2ME2 transformed the keloid fibroblasts into the apoptotic form, such as cell shrinkage, nuclear fragmentation, chromosomal DNA fragmentation, and chromatin condensation, and stained them a brown color.

Caspases are a family of protease enzymes playing essential roles in apoptosis and inflammation. For the apoptosis process, caspases are mainly classified as apoptosis initiators (caspase-8, caspase-9) and executioners (caspase-3) [34]. According to our results, the levels of key apoptosis factors, including caspase-3, caspase-8, and caspase-9, were significantly increased after 24 h of 2ME2 treatment, while the promoting effect of 2ME2 was inhibited by the caspase inhibitor, indicating that 2ME2 may exert its effect through a caspase-dependent mechanism in keloid fibroblasts. During the apoptosis process, one pair of proteins is of great importance: the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. These two proteins usually exist in a heterodimer form, and the Bcl-2/Bax ratio determines the apoptotic fate of cells [35]. According to our results, 2ME2 increased the expression of both Bcl-2 and Bax proteins; however, the Bcl-2/Bax ratio was significantly decreased compared with that in the other groups, which leads to apoptosis of fibroblasts. Cyt-c release is also one of the necessary activities during the apoptosis process, and it was induced by the assembled Bax on the mitochondrial membrane [2]. In group 2ME2, cyt-c

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expression was increased and this might be the indirect result of high expression of assembled Bax after 2ME2 treatment. No significant differences in Bcl-2/Bax ratio and other key apoptosis factors were found between groups S and K, indicating a similar apoptosis level in normal skin and keloid tissue at the cellular level. In addition, expression levels of all factors in group DMSO were quite similar to those in group K, implying that 0.7% DMSO may exert minimal influence on keloid fibroblasts and may hardly affect cell activity.

Drug toxicity is one of the most important indices to evaluate the safety. In this study, a certain concentration of 2ME2 was also used in normal skin fibroblasts at different time points. The results showed that 2ME2 could affect the cell activity of normal fibroblasts from 6 h to 12 h, and after using 2ME2 for more than 12 h, the cell activity of normal fibroblasts was maintained at a high level and it was slightly changed. However, the activity of keloid fibroblasts started to decrease. In such a case, 2ME2 showed the characteristic of selectivity between normal fibroblasts and keloid fibroblasts. Previous studies have also demonstrated similar results, and they showed that 2ME2 could decrease cell viability in nasopharyngeal carcinoma cells, ovarian cancer cells, or human neuroblastoma cells but not in their normal counterparts [36-38].

2ME2, considered to be a new type of anti-cancer drug, showed effects such as the inhibition of proliferation and the promotion of apoptosis in keloid fibroblasts. Other studies also showed the other characteristics of 2ME2 in keloid tissue. Ma [39] demonstrated that the hypoxia/HIF-1 α microenvironment provides a favorable environment for keloid-derived keratinocytes to adopt a fibroblast-like appearance through epithelial-mesenchymal transition. 2ME2 is also regarded as the inhibitor of HIF-1 α , which implies that 2ME2 may ameliorate the hypoxia environment and reverse the EMT process in keloid tissue. Long [10] pointed out that 2ME2 effectively inhibited the protein expression of HIF-1 α and significantly increased the radiotherapy sensitivity of keloid fibroblasts. The toxicity of 2ME2 was also studied. Our result illustrates its characteristics of selectivity. In the study by Luc [40], stimulated T-cells treated with 2ME2 were still able to produce normal levels of cytokines and the author speculated

that 2ME2 may lead to an oral immunomodulatory adjunct therapy with a low side effect profile for individuals undergoing transplantation. Although 2ME2 has these benefits, clinical use of 2ME2 is still limited due to its poor water solubility and low bioavailability [41, 42]. However, 2ME2 is easily soluble in DMSO (60 mg/ml), and the amount of DMSO used in this study showed no significant cell toxicity towards both normal and keloid fibroblasts. Experiments suggest that 2ME2 may be rapidly metabolized in the gastrointestinal tract [43]. In keloid treatment, drugs are used via local injection instead of oral usage, and in such a case, 2ME2 may maintain its bioavailability. Based on these studies, we inferred that 2ME2 may be a promising and effective drug for keloid treatment. However, this study only focused on the effect and mechanisms of action of 2ME2 at the cellular level. Animal experiments still need to be performed to verify its effects *in vivo*.

Disclosure of conflict of interest

None.

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References

- [1] Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L and Kroemer G. Cell death modalities: classification and pathophysiological implications. *Cell Death Differ* 2007; 14: 1237-1243.
- [2] Brenner D and Mak TW. Mitochondrial cell death effectors. *Curr Opin Cell Biol* 2009; 21: 871-877.
- [3] Chalah A and Khosravi-Far R. The mitochondrial death pathway. *Adv Exp Med Biol* 2008; 615: 25-45.
- [4] Pribluda VS, Gubish ERJ, Lavallee TM, Treston A, Swartz GM and Green SJ. 2-Methoxyestradiol: an endogenous antiangiogenic and antiproliferative drug candidate. *Cancer Metastasis Rev* 2000; 19: 173-179.
- [5] Parada-Bustamante A, Valencia C, Reuguen P, Diaz P, Rincion-Rodriguez R and Orihuela PA. Role of 2-methoxyestradiol, an endogenous estrogen metabolite, in health and disease. *Mini Rev Med Chem* 2015; 15: 427-438.

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- [6] Lee JS, Ahn C, Kang HY and Jeung EB. Effect of 2-methoxyestradiol on SK-LMS-1 uterine leiomyosarcoma cells. *Oncol Lett* 2017; 14: 103-110.
- [7] Massaro RR, Faião-Flores F, Rebecca VW, Sandri S, Alves-Fernandes DK, Pennacchi PC, Smalley KSM and Maria-Engler SS. Inhibition of proliferation and invasion in 2D and 3D models by 2-methoxyestradiol in human melanoma cells. *Pharmacol Res* 2017; 119: 242-250.
- [8] Mo JH, Kim JH, Lim DJ and Kim EH. The role of hypoxia-inducible factor 1 α in allergic rhinitis. *Am J Rhinol Allergy* 2014; 28: e100-e106.
- [9] Reumann S, Shogren KL, Yaszemski MJ and Maran A. Inhibition of autophagy increases 2-methoxyestradiol-induced cytotoxicity in SW-1353 chondrosarcoma cells. *J Cell Biochem* 2016; 2016: 3.
- [10] Long F, Si L, Long X, Yang B, Wang X and Zhang F. 2ME2 increase radiation-induced apoptosis of keloid fibroblasts by targeting HIF-1 α in vitro. *Australas J Dermatol* 2016; 57: e32-e38.
- [11] Marneros AG and Krieg T. Keloids—clinical diagnosis, pathogenesis, and treatment options. *J Dtsch Dermatol Ges* 2004; 2: 905-913.
- [12] Nicholas RS, Falvey H, Lemonas P, Damodaran G, Ghanem AM, Selim F, Navsaria H and Myers S. Patient-related keloid scar assessment and outcome measures. *Plast Reconstr Surg* 2012; 129: 648-656.
- [13] Zhang MZ, Dong XH, Guan EL, Si LB, Zhuge RQ, Zhao PX, Zhang X, Liu MY, Adzavon YM, Long X, Qi Z and Wang X. A comparison of apoptosis levels in keloid tissue, physiological scars and normal skin. *Am J Transl Res* 2017; 9: 5548-5557.
- [14] Appleton I, Brown NJ and Willoughby DA. Apoptosis, necrosis, and proliferation: possible implications in the etiology of keloids. *Am J Pathol* 1996; 149: 1441-1447.
- [15] Vangipuram M, Ting D, Kim S, Diaz R and Schüle B. Skin punch biopsy explant culture for derivation of primary human fibroblasts. *J Vis Exp* 2013; 77: e3779.
- [16] Green DR. Means to an end: apoptosis and other cell death mechanisms. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2011.
- [17] Wu CC and Bratton SB. Regulation of the intrinsic apoptosis pathway by reactive oxygen species. *Antioxid Redox Signal* 2013; 19: 546-558.
- [18] Kulikov AV, Shilov ES, Mufazalov IA, Gogvadze V, Nedospasov SA and Zhivotovsky B. Cytochrome c: the Achilles' heel in apoptosis. *Cell Mol Life Sci* 2012; 69: 1787-1797.
- [19] Graf RP, Keller N, Barbero S and Stupack D. Caspase-8 as a regulator of tumor cell motility. *Curr Mol Med* 2014; 14: 246-254.
- [20] Jiang X and Wang X. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem* 2000; 275: 31199-31203.
- [21] Rodriguez J and Lazebnik Y. Caspase-9 and Apaf-1 form an active holoenzyme. *Genes Dev* 1999; 13: 3179-3184.
- [22] Choudhary GS, Al-Harbi S and Almasan A. Caspase-3 activation is a critical determinant of genotoxic stress-induced apoptosis. *Methods Mol Biol* 2015; 1219: 1-9.
- [23] Zhu BT and Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* 1998; 19: 1-27.
- [24] Mueck AO and Seeger H. 2-Methoxyestradiol—biology and mechanism of action. *Steroids* 2010; 75: 625-631.
- [25] Schumacher G, Kataoka M, Roth JA and Mukhopadhyay T. Potent antitumor activity of 2-methoxyestradiol in human pancreatic cancer cell lines. *Clin Cancer Res* 1999; 5: 493-499.
- [26] Dubery RK, Gillespie DG, Keller PJ, Imthum B, Zacharia LC and Jackson EK. Role of methoxyestradiols in the growth inhibitory effects of estradiol on human glomerular mesangial cells. *Hypertension* 2002; 39: 418-424.
- [27] Arbiser JL, Panigrathy D, Klauber N, Rupnick M, Flynn E, Udagawa T and D'Amato RJ. The antiangiogenic agents TNP-470 and 2-methoxyestradiol inhibit the growth of angiosarcoma in mice. *J Am Acad Dermatol* 1999; 40: 925-929.
- [28] Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP and Schweigerer L. The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumor growth. *Nature* 1994; 368: 237-239.
- [29] LaVallee TM, Zhang XH, Johnson MS, Herbstritt CJ, Swartz G, Williams MS, Hembrough WA, Green SJ and Pribluda VS. 2-methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway. *Cancer Res* 2003; 63: 468-475.
- [30] Klauber N, Parangi S, Flynn E, Hamel E and D'Amato RJ. Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. *Cancer Res* 1997; 57: 81-86.
- [31] Moldovan GL, Pfander B and Jentsch S. PCNA, the maestro of the replication fork. *Cell* 2007; 129: 665-679.
- [32] Wang Y, Chen T, Huang H, Jiang Y, Yang L, Lin Z, He H, Liu T, Wu B, Chen J, Kamp DW and Liu G. miR-363-3p inhibits tumor growth by targeting PCNA in lung adenocarcinoma. *Oncotarget* 2017; 8: 20133-20144.
- [33] Hu L, Li HL, Li WF, Chen JM, Yang JT, Gu JJ and Xin L. Clinical significance of expression of proliferating cell nuclear antigen and E-cadherin

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- in gastric carcinoma. *World J Gastroenterol* 2017; 23: 3721-3729.
- [34] Galluzzi L, López-Soto A, Kumar S and Kroemer G. Caspases connect cell-death signaling to organismal homeostasis. *Immunity* 2016; 44: 221-231.
- [35] Ayatollahi SA, Ajami M, Reyhanfard H, Asadi Y, Nassiri-Kashani M, Firoozabadi MR, Davoodi SH, Habibi E and Pazoki-Toroudi H. Bcl-2 and Bax expression in skin flaps treated with finasteride or azelaic acid. *Iran J Pharm Res* 2012; 11: 1285-1290.
- [36] Ting CM, Lee YM, Wong CK, Wong AS, Lung HL, Lung ML, Lo KW, Wong RN and Mak NK. 2-Methoxyestradiol induces endoreduplication through the induction of mitochondrial oxidative stress and activation of MAPK signaling pathways. *Biochem Pharmacol* 2010; 79: 825-841.
- [37] Kato S, Sadarangani A, Lange S, Delpiano AM, Vargas M, Brañes J, Carvajal J, Lipkowitz S, Owen GI and Cuello MA. 2-methoxyestradiol mediates apoptosis through caspase-dependent and independent mechanisms in ovarian cancer cells but not in normal counterparts. *Reprod Sci* 2008; 15: 878-894.
- [38] Zhang Q, Ma Y, Cheng YF, Li WJ, Zhang Z and Chen SY. Involvement of reactive oxygen species in 2-methoxyestradiol-induced apoptosis in human neuroblastoma cells. *Cancer Lett* 2011; 313: 201-210.
- [39] Ma X, Chen J, Xu B, Long X, Qin H, Zhao RC and Wang X. Keloid-derived keratinocytes acquire a fibroblast-like appearance and an enhanced invasive capacity in a hypoxic microenvironment in vitro. *Int J Mol Sci* 2015; 35: 1246-1256.
- [40] Luc JG, Paulin R, Zhao JY, Freed DH, Michalakis ED and Nagendran J. 2-Methoxyestradiol: a hormonal metabolite modulates stimulated T-cells function and proliferation. *Transplant Proc* 2015; 47: 2057-2066.
- [41] Cho JK, Hong KY, Park JW, Yang HK and Song SC. Injectable delivery system of 2-methoxyestradiol for breast cancer therapy using biodegradable thermosensitive poly (organophosphazene) hydrogel. *J Drug Target* 2011; 19: 270-280.
- [42] Verenich S and Gerk PM. Therapeutic promises of 2-methoxyestradiol and its drug disposition challenges. *Mol Pharm* 2010; 7: 2030-2039.
- [43] Kambhampati S, Rajewski RA, Tanol M, Hague I, Das A, Banerjee S, Jha S, Burns D, Borrego-Diaz E, Veldhuizen PJV and Banerjee SK. A second-generation 2-methoxyestradiol prodrug is effective against Barrett's adenocarcinoma in a mouse xenograft model. *Mol Cancer Ther* 2013; 12: 255-263.