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Effects of Luteolin on the Proliferation of Rat Lung Fibroblasts

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[ABSTRACT] AIM: To approach the effects of luteolin on proliferation of rat lung fibroblasts. **METHODS:** The lung fibroblasts were isolated from the lungs of Sprague-Dawley rats at postnatal day 3, and were then passaged to 96-well culture plates. All cells were divide into 6 groups including normal control group, positive control group, 4×10 time, the cytomorphology of LFb, drew growth curve, determined superoxide dismutase (SOD) activity were observed. **RESULTS:** Luteolin at the concentration of 4×10^{-4}, 4×10^{-5} mol·L^{-1} inhibited evidently the growth and proliferation of lung fibroblasts, and enhanced SOD activity. The density, interval and volume of lung fibroblasts became decrescent. The effects, dose-dependently, had obvious statistical significance compared with normal control group. **CONCLUSION:** Luteolin can inhibit the proliferation of the lung fibroblasts in rat.

[KEY WORDS] Luteolin; Lung fibroblasts; Proliferation inhibition

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1 Introduction

Luteolin, chemical name 4*H*-1-benzopyran-4-one, 2-(3, 4-dihydroxy-phenyl)- 5,7-dihydroxy, is an active component of *Ajuga decumbens and Dracocephalum integrifolium*, and has multi-pharmacological effects including antibiosis, anticancer, spasmolysis, eliminating phlegm, inhibiting enzyme, diuresis and choleretic effect and so on. In recent studies, it was reported that "amyotrophic lateral sclerosis" and severe acute respiratory syndrome (SARS) can be treated by Luteolin. We have studied Luteolin for years and have found that transforming growth factor-β (TGF-β) of lung tissue, generally residing in animal's normal cells (including Lung Fibroblasts, LFb) and transformant, can be inhibited by Luteolin [1,2]. However, the main histopathological characteristics of pulmonary fibrosis (PF) are diffuse alveolitis early, pathological proliferation of large numbers of LFb laterly and progressive accumulation of collagens, resulting in impaired lung function and respiratory failure. Thereby, inhibiting excess proliferation of LFb is a vital treatment means of pulmonary fibrosis. In this article, effects of luteolin on LFb proliferation were mainly discussed so as to provide new therapeutic approaches to pulmonary fibrosis.

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2 Instruments and Agents

2.1 *Agents*

Luteolin, the purity being more than 99%, was purchased from Hangzhou Forster Chemicla Co. Ltd (batch No. 080628); trypsin and tetrazolium were purchased from Amresco (USA), (Batch No. 8C038D16 and 2010/08).

2.2 *Instruments*

Bio-Tek instruments were purchased from Microplate Manager4.0 Bio-rad Laboratories.

2.3 *Animals*

Experiments were performed on Sprague-Dawley rats at postnatal day 3, from Laboratory Animal Center of Nanjing Medical University, certificate of animal: SCXK (Su)2002-0031.

3 Methods

3.1 *Preparation of LFb*

According to literature [3, 4], fibroblasts were isolated from the lungs of Sprague-Dawley rats at postnatal day 3. In brief, lungs were removed, trimmed of extraneous tissue, minced, subjected to trypsin digestion for 40 min and centrifugated (1 000 r·min^{-1} for 10 min), and resuspended in complete medium containing DMEM medium, 10% (*V*/*V*) calf serum, penicillin (10000 U/100mL), streptomycin (10 mg·mL^{-1}). The cell suspension was plated for 24 h and

then the culture flasks were rinsed with phosphatic balanced salt solution (PBSS) to remove nonadherent cells and cultured in complete medium until 90%~95% confluent. The isolated cells were determined to be predominantly fibroblasts (99%) based on their morphological appearance by phase-contrast microscopy. Cells taken between the 3rd and the 4th passages were used for the experiments.

3.2 *Identification of morphology of LFb*

The morphology of LFb was Fusiform-like, and the cells had features that they had longer cell pustute and orbicular-ovate nucleus in the center of LFb and actiniform or paliform arrangement.

3.3 *Groups*

The LFb were randomly divided into 6 groups, 8 repeated wells in each, containing normal control group, positive control group, 4 test groups of Luteolin (4×10^{-4}, 4×10^{-5}, 4×10^{-6}, 4×10^{-7} mol·L^{-1}).

3.4 *Determination of LFb proliferation*

3.4.1 *Determination of Growth curve of LFb* Chromometry of MTT [5, 6]: confluent LFb, the 2nd generation, were passaged to 96-well plates at a density of 5×10^4 cells/well, allowed to adhere for 24 h in DMEM complete medium at 37 °C in 5% CO_2, and then were serum-starved overnight to synchronize cell cycle stage. The normal control group received DMEM complete medium containing DMEM medium, 0.4% (*V*/*V*) calf serum, penicillin (10 000 U/100 mL), streptomycin (10 mg·mL^{-1}). The different concentrations of Luteolin were added to test groups. Starting on day 2, *OD* of every group were recorded continuously by the multi-scanning spectrophotometer with wavelength at 490 nm for 7 d, drew growth curve.

3.4.2 *Determination of LFb proliferation* According to literature [7,8], confluent LFb, the second generation, were passaged to 96-well plates at a density of 2.5×10^4 cells/well, allowed to adhere for 24 h in complete medium at 37°C in 5% CO_2 , removed complete medium, and randomly divided into 6 groups described previously. Every group which was received DMEM serum-free medium to serum-starve to synchronize cell cycle stage was incubated for 24 h, and then DMEM serum-free medium was removed from every well. The normal control group received again DMEM serum-free medium, the positive control group received DMEM serum-free medium and Prednisolone acetate, 4 Luteolin test groups (with LFb) received DMEM serum-free medium and Luteolin (4×10^{-4}, 4×10^{-5}, 4×10^{-6}, 4×10^{-7} mol·L^{-1}). Every group was incubated for 24 , 48, 72 h in incubator at 37°C in 5% CO_2. At the corresponding time point, 20 μL supernatant fluid was removed from each well, adding 20 μL MTT, continuing to incubate for 4 h, removing again cell culture fluid, adding 150 μL DMSO into each well, after incubating for 15 min, shaking the plate lightly to make ianthinus crystal dissolve well, recording *OD* on the multi-scanning spectrophotometer with wavelength at 490 nm.

3.5 *Determination of SOD activity of LFb*

The 2nd generation confluent LFb cultures were passaged to 96-well plates at a density of 5×10^5 cells/well, being allowed to adhere for 24 h in complete medium at 37°C in 5% CO_2, and then removed complete medium. All cells were randomly divided into 6 groups each of which received DMEM serum-free medium to serum-starve to synchronize cell cycle stage. After incubation for 24 h, DMEM serum-free medium was removed from each well .The normal control group received again DMEM serum-free medium, the positive control group received DMEM serum-free medium and Prednisolone acetate. 4 Luteolin test groups (with LFb) received DMEM serum-free medium and Luteolin (4×10^{-4}, 4×10^{-5}, 4×10^{-6}, 4×10^{-7} mol·L^{-1}). Every group, being incubated for 24 , 48, 72 h at 37°C in 5% CO_2, at the corresponding time point ,was freeze-thawed repeatedly, disrupted, shaken well and centrifugated 4 000 r·min^{-1} for 15min using refrigerated centrifuge, removing supernatant fluid. SOD activity was determined using SOD kit according to the manufacturers' protocol. Then *OD* was recorded by the multi-scanning spectrophotometer with wavelength at 550 nm.

3.6 *Statistics*

All data were expressed as means ± standard deviation ($\bar{x} \pm s$). The statistical significance of differences was analyzed using Student's *t*-test with a significance level of $P < 0.05$ and $P < 0.01$.

4 Results

4.1 *Effects of Luteolin on morphology of LFb*

Fig. 1 showed the LFb stimulated by Luteolin for 48, 72 h at the concentrations of 4×10^{-5}, 4×10^{-6}, 4×10^{-4} mol·L^{-1} was Fusiform-like and actiniform or paliform arrangement with broader cells compartment. In test groups of Luteolin, LFb of which the number reduced obviously had no distinguished morphological difference compared with the normal control group. This result suggested that Luteolin at the concentration of 4×10^{-5}, 4×10^{-6}, 4×10^{-4} mol·L^{-1} could obviously inhibit LFb proliferation.

4.2 *Effects of Luteolin on growth curve of LFb*

Fig.2 showed that when Luteolin at 4×10^{-4}, 4×10^{-5} or 4×10^{-6} mol·L^{-1}, *OD* were obviously lower ($P < 0.01$) compared with normal control group and was dose-dependent. This result suggested that experimental concentration of Luteolin can inhibit LFb proliferation (Fig. 2).

4.3 *Effects of Luteolin on LFb proliferation*

Table 1 provided further support that Luteolin can inhibit LFb proliferation. LFb proliferation was obviously inhibited (P <0. 01) by Luteolin at the concentration of 4×10^{-4}, 4×10^{-5},

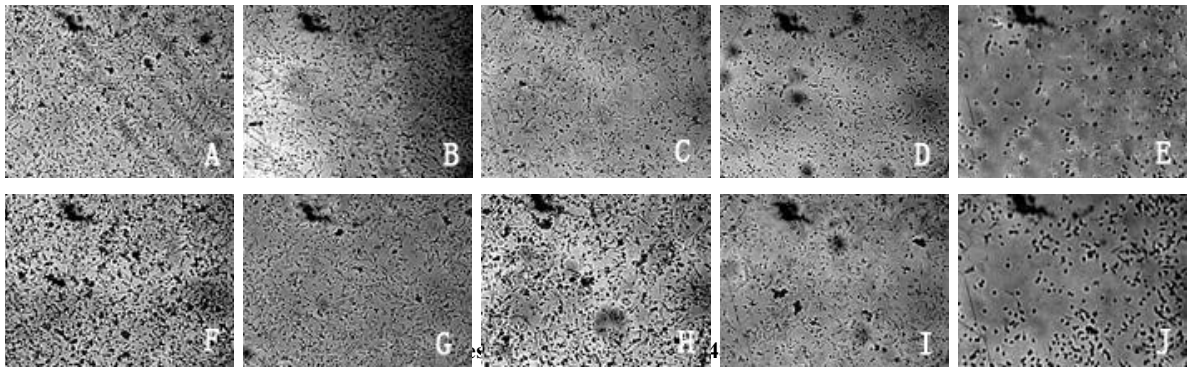

Fig. 1 LFb stimulated by Luteolin

A: Normal control at 48 h; B: Prednisolone acetate at 48 h; C: Luteolin (4×10^{-6} mol·L^{-1}) at 48 h; D: Luteolin (4×10^{-5} mol·L^{-1}) at 48 h; E: Luteolin (4×10^{-4} mol·L^{-1}) at 48 h F: Normal Control at 72 h; G: Prednisolone acetate at 72 h; H: Luteolin (4×10^{-6} mol·L^{-1})at 72 h; I: Luteolin (4×10^{-5} mol·L^{-1}) at 72 h; J: Luteolin (4×10^{-4} mol·L^{-1}) at 72 h

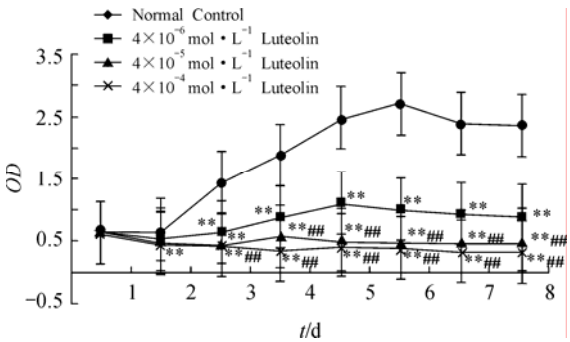

Fig. 2 Effects of Luteolin on growth curve of LFb

$^{**}P < 0.01$ *vs* control group; $^{\#\#}P < 0.01$ *vs* 10^{-6}mol·L^{-1} Luteolin

4×10^{-6} mol·L^{-1} at 48, 72 h compared with normal control group, and this effect was dose-dependent. Especially, 4×10^{-4} mol·L^{-1} with complete proliferation-inhibiting effect on LFb showed no notable difference ($P > 0.05$) compared with quiescent LFb made by incubation in defined serum-free medium. However, Luteolin, 4×10^{-7} mol·L^{-1}, showed no obvious effect on LFb proliferation compared with normal control group. The effect of prednisolone acetate on LFb proliferation had statistical difference ($P < 0.05$) compared with normal control group. At 24 h, except positive control group, there was no obvious difference between Luteolin groups and normal control group (Table 1).

Table 1 Effects of Luteolin on LFb proliferation ($\bar{x} \pm s$, n=8)

Group c(mol·L^{-1})		6 h (OD_{490})	24 h (OD_{490})	48 h (OD_{490})	72 h (OD_{490})
Normal Control		0.214±0.96	0.554±0.101	0.655±0.139	0.795±0.049
Prednisolone Acetate	4×10^{-5}	0.213±0.88	0.352±0.056**	0.519±0.089**	0.571±0.121**
Luteolin	4×10^{-4}	0.218±0.87	0.535±0.121	0.464±0.130**	0.531±0.099**
Luteolin	4×10^{-5}	0.220±0.94	0.543±0.307	0.513±0.290**	0.596±0.087**
Luteolin	4×10^{-6}	0.222±1.10	0.545±0.317	0.590±0.130**	0.679±0.200**
Luteolin	4×10^{-7}	0.216±0.89	0.541±0.320	0.646±0.220	0.743±0.162

** $P < 0.01$ *vs* control group

4.4 *Effects of Luteolin on SOD activity of LFb*

Table 2 indicated that SOD activity of Prednisolone acetate and Luteolin groups at 48, 72 h had statistical significance ($P < 0.01$) compared with the normal control group. Luteolin at the concentration of 4×10^{-4} mol·L^{-1} at 48 h enhanced SOD activity by 59% relatively to the normal control group. At 24 h, except positive control group, there was no obvious difference between Luteolin groups and normal control group (table 2).

5 Discussion

Alteration of fibration in the lung tissue can promote inflammation of lung mesenchymal cells and lead to pulmonary artery hypertension [9], and even lead to significant morbidity and mortality of lung cancer. A series of enzymes and cytokines in LFb, inhibited by some drugs and factors, can inhibit cell proliferation by regulating the growth cycle of LFb [10].

LFb was characterized by being cultured easily with-stronger survivability to some inductors and being identified easily after transformation, thereby LFb was taken as target cell to research effects of Luteolin on proliferation、morphology and biochemical indicators of LFb. Our experimental results showed that cytostatic effect of Luteolin on LFb was dose-dependent; therefore we concluded that Luteo-

lin possibly had certain therapeutic effect on pulmonary fibrosis. At 24 h, the effects of Luteolin on LFb proliferation had no statistical significance, so we concluded that the time when Luteolin produced inhibiting effect on LFb exceeded 24 h. In recent studies [11], pulmonary fibrosis, correlating intimately to oxidative-stress reaction, was also treated with antioxidant. Our findings were reported here that in this culture system, SOD activity was enhanced by Luteolin, suggesting that the inhibiting effect of Luteolin on LFb was possibly related to antioxidative effect of Luteolin. Despite our findings, the precise mechanisms of Luteolin underlying the treatment of pulmonary fibrosis are still unknown. Further studies are to be undertaken.

Table 2 Effects of Luteolin on SOD activity of LFb ($\bar{x}$ ±s, *n*=8)

Group	*c* (mol·L^{-1})	24 h SOD (U·mL^{-1})	48 h SOD (U·mL^{-1})	72 h SOD (U·mL^{-1})
Normal Control		22.3±1.1	24.7±0.92	26.5±0.49
Prednisolone acetate	4× 10^{-5}	25.9±1.1*	29.5±0.75**	34.1±0.82**
Luteolin	4× 10^{-4}	22.8±1.0	30.4±0.30**	42.3±0.56**
Luteolin	4× 10^{-5}	23.4±0.86	29.7±0.20**	36.2±1.5**
Luteolin	4× 10^{-6}	23.9±0.95	28.5±0.63**	33.4±1.3**
Luteolin	4× 10^{-7}	23.8±1.2	27.5±0.37*	33.0±0.52**

*P<0.05, ** P<0.01 *vs* control group

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