

## Effects of Luteolin on Fetal Bovine Serum-induced Events in Cultured Rat Vascular Smooth Muscle Cells

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Cell cycle activation and progression in vascular proliferative disease represent potent therapeutic targets. Luteolin, which occurs as glycosylated forms in celery, green pepper, perilla leaf, and camomile tea, has demonstrated antimutagenic, antitumorigenic, antioxidant, and antiinflammatory properties. In this study, we investigated the effect of luteolin on the proliferation of primary cultured rat aortic vascular smooth muscle cells induced by 5% fetal bovine serum. Luteolin at concentrations of 5, 20, and 50  $\mu\text{M}$  significantly inhibited this proliferation by 29.6, 50.8, and 83.1%, respectively. The incorporation of [ $^3\text{H}$ ]-thymidine into DNA was also inhibited by 25.8, 57.6, and 81.0%, respectively. Flow cytometry analysis of DNA content revealed that FBS-inducible cell cycle progression was blocked by luteolin. Luteolin showed no cytotoxicity in VSMCs in this experimental condition according to WST-1 assays. Luteolin may represent a potential anti-proliferative agent for treatment of angioplasty restenosis and atherosclerosis.

**Key words** : Luteolin, atherosclerosis, proliferation, vascular smooth muscle cell (VSMC)

### Introduction

Vascular smooth muscle cells (VSMCs) in the arterial media are fully differentiated to play their physiological roles as regulators of vascular wall tension. They proliferate at low indices or persist in the G0 phase of the cell cycle [1,5]. Proliferation of VSMCs occurs in response to arterial injury and plays a crucial role in the pathogenesis of vascular disease, such as atherosclerosis, hypertension and restenosis [8]. An alternative approach to preventing the proliferation and migration of VSMCs may be to influence the downstream intracellular signaling events responsible for transducing the signals from the various growth factor receptors.

Flavonoids are naturally occurring polyphenolic compounds presented in a variety of fruits, vegetables and seeds. Flavonoids have many biological and pharmacological activities including antioxidative, antiinflammatory and anti-

tumor effects [4]. Luteolin (Fig. 1), a polyphenolic compound available in foods of plant origin, belongs to the flavone subclass of flavonoids, usually occurring as glycosylated forms in celery, green pepper, perilla leaf and camomile tea [9,10]. It has been reported to display antimutagenic, antiplatelet aggregation and anticancer effects [2,7].

In the present study, we investigated the antiproliferative effects of luteolin on primary cultured VSMCs stimulated by the administration of fetal bovine serum (FBS). Our results reveal that luteolin is a potential agent for the treatment of vascular disorders like atherosclerosis.

### Materials and Methods

#### Chemicals and reagents

Luteolin (Sigma Chemical Co., MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and further diluted in Dulbecco's Modified Eagle Medium (DMEM) without FBS. The cell culture materials were obtained from Gibco-BRL (Rockville, MD, USA), and other chemical reagents were from Sigma Chemical Co. [ $^3\text{H}$ ]-thymidine was purchased Amersham Pharmacia Biotech (Buckinghamshire, UK). Other chemicals were of analytical grade.

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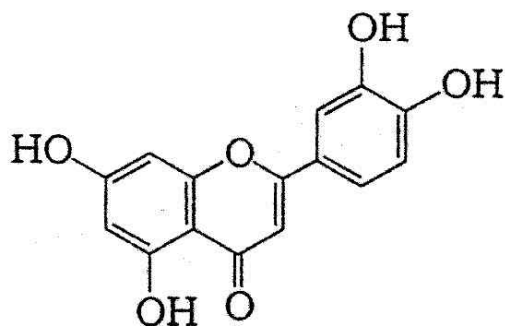


Fig. 1. Chemical structure of luteolin

#### Cell culture

Male Sprague-Dawley rats (Samtako Bio Korea Co., Ltd., Osan, Korea) weighing around 280 g were fed a normal chow diet and given water ad libitum. VSMCs were isolated by enzymatic dispersion as previously described [6] according to the modified method of Chamley *et al.* [3]. Cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> incubator. The purity of VSMCs culture was confirmed by immunocytochemically based on α-smooth muscle actin localization. The passage of VSMCs used in this experiment was 5-9.

#### Cell counts

VSMCs were seeded onto 12-well culture plates at  $1 \times 10^5$  cells/ml, and then cultured in DMEM containing 10% FBS at 37°C for 24 hr. Under these conditions, a cell confluence of 70% was reached. The medium was then replaced with serum-free medium containing luteolin (5, 20 or 50 µM), and cells were then stimulated with 5% FBS and trypsinized using trypsin-EDTA, and counted using a hemocytometer.

#### DNA synthesis by [<sup>3</sup>H]-thymidine incorporation

For [<sup>3</sup>H]-thymidine incorporation experiments, VSMCs were seeded onto 24-well culture plates using conditions identical to those described above, and 1 µCi/ml of [<sup>3</sup>H]-thymidine was added to medium. Reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes with phosphate-buffered saline (PBS) containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v) on ice. The acid-insoluble [<sup>3</sup>H]-thymidine was extracted into 250 µl of 0.5 M NaOH/well, and this solution was mixed with a 3 ml scintillation cocktail (Ultimagold,

Packard Bioscience, CT, USA). The mixed solution was quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany), and 50 µl of residual solution was measured using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

#### Cell viability assay

For WST-1 assay (Premix WST-1, Takara, Japan), VSMCs were seeded in 96-well plates at  $8 \times 10^3$  cells/well and pre-cultured in serum-free medium in the presence or absence of luteolin compounds for 24 hr. WST-1 reagent was added at 20 hr and further incubated for 4 hr. Then the absorbance was determined in an ELISA at a wavelength of 450 nm.

#### Cell cycle progression analysis

To estimate the proportion of cells in the various phases of the cell cycle, cellular DNA contents were measured by flow cytometry (FACS). Cells were harvested, fixed in 70% ethanol, and stored at -20°C. Cells were then washed twice with ice-cold PBS and incubated with RNase and propidium iodide (PI), a DNA-intercalating dye. Cell cycle phase analysis was performed using the FACSCalibur (Becton-Dickinson Co., San Jose, USA) and the proportion of cells within the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases was determined by analysis with ModFit LT V2.0 (Verity Software House, Topsham, USA).

#### Statistical analysis

The experimental results were expressed as mean±S.E.M. A one-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett. Differences with  $p < 0.05$  were considered statistically significant.

## Results

#### Effect of luteolin on the proliferation of VSMCs

The inhibitory effect of luteolin on proliferation of FBS-induced VSMCs was measured by cell number. The number of cells was significantly increased by 5% FBS for 24 hr. The FBS-induced cell numbers were significantly decreased by pre-treatment of luteolin for 24 hr in a concentration-dependent manner. The percentage of inhibition exerted by 5, 20 and 50 µM luteolin were 29.6, 50.8 and 83.1% respectively (Fig. 2).

Effect of luteolin on the DNA synthesis of VSMCs

The effect of luteolin on the DNA synthesis of VSMCs was examined. Stimulation of VSMCs by 5% FBS potently increased [<sup>3</sup>H]-thymidine incorporation from 1,803 to 15,979 cpm/well. luteolin significantly inhibited the FBS-induced DNA synthesis in a concentration-dependent manner, and the inhibition percentages were 25.8, 57.6 and 81.0% at the concentrations of 5, 20 and 50 μM, respectively (Fig. 3). No significant difference in viability was detected between control cells and those treated with luteolin (5-50 μM) when examined by WST-1 assay (Fig. 4).

Effect of luteolin on cell cycle progression

Flow cytometric analysis (Table 1) demonstrated that luteolin affects cell cycle progression induced by 5% FBS. A primary culture of VSMCs was subjected to serum-deprivation for 24 hr, which resulted in approximately 87.8±0.75% of cells being synchronized in the G0/G1 phase. After incubation for 24 hr in the presence of FBS, the percentage of cells in S phase increased from 0.9±0.3 to 18.2±1.3%. In contrast, luteolin (5-50 μM) blocked FBS-induced cell cycle progressions in a concentration-dependent manner (Table. 1). Luteolin (5, 20 and 50 μM) reduced the percentage of FBS-stimulated cells in S phase to 14.2±1.2% (*p*<0.05), 9.0±0.4% (*p*<0.01) and 3.5±0.6% (*p*<0.01), respectively (Table. 1;

*n*=3, duplicate cultures). This finding indicates that luteolin must act during early events in the cell cycle, since it is effective against DNA synthesis. Luteolin arrested significant numbers of cells in the G1 phase of the cell cycle, suggesting that its anti-proliferative effects in VSMCs are due to cell cycle arrest.

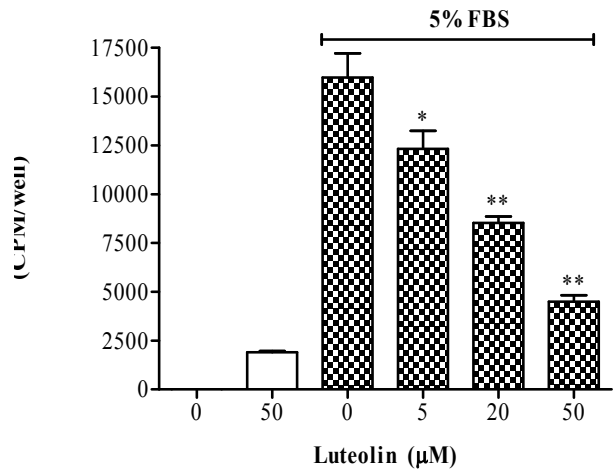


Fig. 3. Effect of luteolin on FBS-induced DNA synthesis. VSMCs were pre-cultured in serum-free medium at different concentrations (5, 20 and 50 μM) of luteolin for 24 hr, and then exposed to 5% FBS for 24 hr. [<sup>3</sup>H]-thymidine incorporation assay was performed. Data are expressed as mean±S.E.M. from four different sets of experiments \**p*<0.05, \*\**p*<0.01 vs. FBS- treated VSMCs.

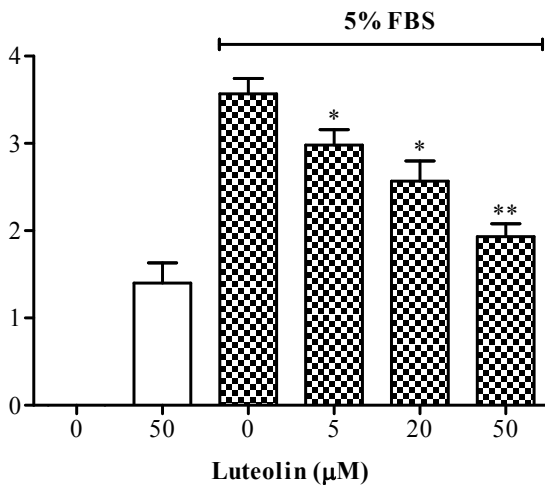


Fig. 2. Effect of luteolin on the FBS-induced VSMC proliferation. VSMCs were pre-cultured in serum-free medium at different concentrations (5, 20 and 50 μM) of luteolin for 24 hr, and then exposed to 5% FBS for 24 hr. Data are expressed as mean±S.E.M. from four different sets of experiments \**p*<0.05, \*\**p*<0.01 vs. FBS-treated VSMCs.

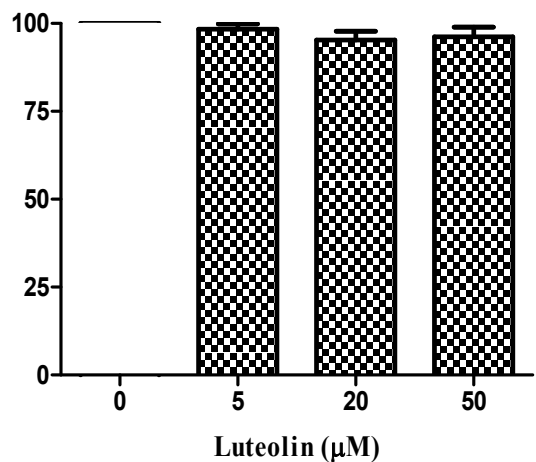


Fig. 4. Effect of luteolin on VSMCs viability. VSMCs were pre-cultured in serum-free medium at of different (5, 20 and 50 μM) concentrations of luteolin. After 24 hr, the cells were transferred to a hemocytometer. WST-1 assay was performed as described in materials and methods.

Table 1. Effect of luteolin on FBS-stimulated cell cycle progression

	G0/G1	S	G2/M
Control	87.8±0.7	0.9±0.3	11.3±0.5
Luteolin (50 µM)	87.4±0.2	1.0±0.4	11.6±0.6
FBS	70.0±0.9 <sup>#</sup>	18.2±1.3 <sup>#</sup>	11.8±1.5
FBS + Luteolin (5 µM)	73.5±1.2 <sup>*</sup>	14.2±1.2 <sup>*</sup>	12.3±0.9
FBS + Luteolin (20 µM)	78.4±1.2 <sup>**</sup>	9.0±0.4 <sup>**</sup>	12.5±0.4
FBS + Luteolin (50 µM)	83.7±0.7 <sup>**</sup>	3.5±0.6 <sup>**</sup>	2.7±0.9

The VSMCs were pre-cultured in the presence or absence of luteolin (5-50 µM) in serum-depleted medium for 24 hr, and then VSMCs were stimulated by 5% FBS. After 24 hr, individual nuclear DNA content was reflected by fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment, where data from at least 10,000 events were obtained. Results are means±SD, n=3. <sup>#</sup> $p < 0.01$  vs. FBS-unstimulated cells (Control). <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  vs. without luteolin in FBS-stimulated cells.

### Discussion

Many growth factors induce the proliferation of VSMCs *in vitro* and *in vivo*. Among them, FBS and basic fibroblast growth factor are important regulators of VSMC behavior through their well-defined actions as potent chemo-attractants and strong mitogens. Administration of these growth factors enhances intimal thickening after angioplasty in the rat, whereas injection of antibodies or use of antisense technology to block signal transduction by either of these growth factors potentially inhibits postinjury intimal hyperplasia in the rat and restenosis in the pig, suggesting that VSMC growth plays an important role in these pathogenesis.

The aim of this study was to assess the effect of luteolin on the proliferation of serum-induced VSMCs. The anti-proliferative effects of luteolin on VSMCs were examined by a direct cell counting assay. We found that luteolin is a potent inhibitor of VSMCs proliferation. The 5% FBS-induced VSMCs proliferation was significantly decreased by pre-treatment of luteolin for 24 hr in a dose-dependent manner (Fig. 2). The inhibitory effect of luteolin on incorporation of [<sup>3</sup>H]-thymidine into the cells gradually declined as the delay between the treatments of VSMCs with luteolin and FBS (Fig. 3). In addition, the antiproliferative effect of luteolin on VSMCs was not due to cellular cytotoxicity, which were demonstrated by WST-1 assay (Fig. 4).

Recent studies [3] have emphasized the role of G-S events in the regulation of cell proliferation through complex stimulant and inhibitory signals driven by cyclin-dependent kinases and their inhibitors, respectively. To further investigate the pattern of the antiproliferative effect of luteolin, flow cytometry analysis was performed. As revealed by flow cytometry assay, the antiproliferative effect of luteolin was as-

sociated with an accumulation of cells in G0/G1 phase of the cycle (Table 1). Since the observed accumulation in G0/G1 reflected a specific effect of luteolin on cell cycle progression rather than a decrease of cell number due to apoptosis.

Therefore, these results suggest that anti-proliferating agent could be an useful preventive or therapeutic agent for cardiovascular diseases including atherosclerosis, and luteolin can be one of these candidate agents.

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초록 : 소태아혈청으로 유도된 흰쥐 혈관평활근세포의 luteolin 효과

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혈관 증식 질환에서 세포주기 활성화와 진행은 중요한 치료 목적으로 사용된다. Luteolin는 glycosylated 형태로 샬러리, 후추, 들깨 잎 그리고 카밀레 차에 존재하며 항돌연변이, 항종양, 항산화 그리고 항염증을 나타낸다. 본 연구에서는 흰쥐 동맥으로부터 분리한 혈관평활근세포를 배양하여 소태아혈청으로 유도된 증식에서 luteolin 효과에 대해 조사했다. Luteolin이 5% 소태아혈청으로 유도된 흰쥐의 혈관평활근세포 증식과 DNA 합성을 5, 20 그리고 50  $\mu$ M에서 억제했다. 혈관평활근세포 증식을 각각 29.6, 50.8 그리고 83.1% 억제했고 DNA 합성은 각각 25.8, 57.6 그리고 81.0% 억제했다. 게다가, 유세포분석 결과 소태아혈청으로 유도된 혈관평활근세포의 세포주기는 luteolin에 의해 차단되었다. 이러한 결과는 세포독성에 의해서도 나타날 수 있기 때문에 WST-1 분석으로 세포독성을 확인한 결과 세포독성 없이 세포주기를 차단하는 효과임을 확인했다. 이상의 결과들은 luteolin이 혈관 스펀트와 동맥경화의 치료를 위한 의미있는 항증식 물질임을 보여준다.