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Research Paper

Luteolin inhibited hydrogen peroxide-induced vascular smooth muscle cells proliferation and migration by suppressing the Src and Akt signalling pathways

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Abstract

Objectives Luteolin is a naturally occurring flavonoid found in many vegetables, fruits and medicinal plants. The migration and proliferation of vascular smooth muscle cells (VSMCs) are the critical pathological processes in various cardiovascular diseases, such as atherosclerosis. In this study, we investigated the effect of luteolin and its latent mechanism on the proliferation and migration of VSMCs stimulated by hydrogen peroxide (H_2O_2).

Methods VSMC proliferation and cell viability was assayed using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) method or by cell counting, and H_2O_2-elicited migration of VSMCs was measured using a transwell migration assay. The phosphorylation levels of Src, 3-phosphoinositide-dependent kinase 1 (PDK1) and Akt (protein kinase B) were analysed by immunoblotting.

Key findings This study demonstrated that luteolin showed a particularly inhibitory effect on H_2O_2-elicited VSMC proliferation and migration. In previous research, we originally explored the function of luteolin in blocking H_2O_2-triggered Src and Akt signalling pathways. The activation of Src, PDK1, Akt (308), Akt (473) in the luteolin-treated group was significantly lower than that seen in the H_2O_2 group.

Conclusions These findings strongly suggested that luteolin suppresses H_2O_2-directed migration and proliferation in VSMCs partially due to down-regulation of the Akt and Src signalling pathways, which are important participants in the processes of migration and proliferation of VSMCs.

Introduction

The abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) are strongly implicated as an important component of vessel wall remodelling in response to oxidative stress (e.g. in the development of restenosis after percutaneous coronary intervention as well as in the pathogenesis of hypertension and the progression of atherosclerosis).[1,2] Reactive oxygen species (ROS) are involved in many pathophysiological processes of VSMCs, such as growth, migration, contraction and differentiation.[3–6] There is growing evidence to support the finding that hydrogen peroxide (H_2O_2), itself a ROS in addition to being the downstream product of the dismutase of superoxide, plays an important role in the pathogenesis of several cardiovascular diseases associated with the proliferation and migration of VSMCs.[7–10] Therefore, inhibition of H_2O_2-stimulated VSMC migration and proliferation represents an important target point for therapeutic intervention to attenuate vascular diseases and prevent restenosis after coronary angioplasty.[11]

Luteolin is a common flavonoid present in Chinese herbal medicines such as flos lonicerae, flos chrysanthemi and decumbent bugle herb. It has many pharmacological actions, including antibiotic, anti-inflammatory, antioxidant and anti-cancer activity.[12] Many studies have reported that dietary flavonoids provide protection against VSMC disorders.[13–16] A recent report demonstrated that luteolin inhibits lysophosphatidylcholine (LPC)-induced apoptosis in endothelial cells.[17] This report suggested that luteolin may have a potential effect against atherosclerosis; however, little investigation was made to test the effect of luteolin on VSMC proliferation and migration.

The potential role of H_2O_2 in the pathogenesis of vascular diseases is related to its effect on several signalling protein kinases, such as Akt in VSMCs.[8,18] Akt/protein kinase B (PKB) is a serine/threonine protein kinase and the Akt signalling pathway is involved in the regulation of multiple biological processes, including cell survival, proliferation, migration and glycogen metabolism.[19]Ablation of Akt leads to severe atherosclerosis and occlusive artery disease[20] while the absence of Akt induces plaque vulnerability and cardiac dysfunction in a mouse model of atherosclerosis.[21] Akt is recruited to the cell membrane, where it is activated by the 3-phosphoinositide-dependent kinase 1 (PDK1), which was previously reported to mediate VSMC migration activated by H_2O_2.[22,23]

The nonreceptor tyrosine kinase Src plays a central role in mediating migration and proliferation in cultured VSMCs.[13] Src kinase signalling is involved in the atherogenic responses in VSMCs[24] and blockage of Src activity reduces the proliferative response of VSMCs.[25] Cell migration also depends on Src activity, which has been verified using either a Src inhibitor or anti-Src antibodies.[26]

In this study, we observed the effects of luteolin on the inhibition of H_2O_2-induced VSMC proliferation and migration. Furthermore, we focused our examination on the signalling pathways that are affected by luteolin.

Materials and Methods

Materials

Luteolin (Figure 1) was purchased from Sigma (St Louis, USA). Luteolin was first dissolved in dehydrated alcohol to make 50 mM stock solutions and then serially diluted in phosphate-buffered saline (PBS) immediately before experimental use. Phospho-specific polyclonal antibodies to Akt, PDK1, Src and the anti-Akt polyclonal and anti-Src polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, USA). Transwell chambers were obtained from Corning Incorporated Life Sciences (Lowell, USA).

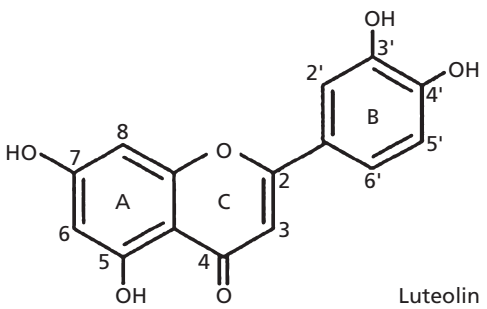

Figure 1 Chemical structure of luteolin (3′,4′,5,7-tetrahydroxyflavone).

Primary vascular smooth muscle cell culture

VSMCs were harvested from rat aortas using the explant technique.[27] Briefly, The thoracic aortas were quickly excised and taken out after sacrifice of Sprague–Dawley rats (8–10 weeks old, male or female, 200 ± 20 g, provided by the Institute of Laboratory Animal of Xuzhou Medical College). All experimental protocols used in this study, particularly in respect to the ethical animal care and use of animals in this study, were approved by the ethical Committee in Xuzhou Medical College. The thoracic aorta of rats were separated carefully and cut into small pieces (approximately 1–2 mm × 1–2 mm) and then these tissue pieces were placed on the wall of a culture bottle. They were cultivated and maintained in Dulbecco's modified eagle medium (DMEM; Hyclone, Logan, UT, USA) with 20% fetal bovine serum (FBS; Gibco/Invitrogen, Grand Island, NY, USA) 1 h later. After being cultivated for one week, many cells had grown on the bottom of the culture bottle and had developed into the typical 'peak and valley' growth pattern. The VSMCs were incubated at 37°C in 5% CO_2 and DMEM containing 10% FBS supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) for subculturing. The purity of VSMCs was identified through the use of a specific antibody against smooth muscle α-actin (DAKO Corp., Glostrup, Denmark). For all experiments, early cell passages 3–5 were used. Cells were grown to 60–70% confluence and then serum-starved for at least 16 h in DMEM containing 5.5 mM glucose and 1% antibiotics before the start of each experiment.

Cell viability/proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was prepared as a 5 mg/ml solution in PBS and filtered through a 0.22 μm filter immediately before use. For measurement of cell viability or proliferation, VSMC suspensions were seeded into 96-well plates at 1×10^4 cells per well 24 h before treatment. Following treatment with luteolin and/or H_2O_2 at indicated concentrations for 48 h, cell viability was determined using the MTT assay. Briefly, 10 μl (5 mg/ml) MTT working solution was added to each well, and after incubation at 37°C for 4 h the MTT solution was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the dark-purple water-insoluble crystals. The absorbance of each well was measured at 570 nm using an EL × 800 Universal Microplate Reader (BIO-TEK, Norcross, GA, USA). The proliferation amount of the control VSMCs was indicated as 100% and results were expressed as relative proliferation. For the cell counting assay, VSMCs (1×10^5/ml) were used in 24-well plates. The number of cells in each well was counted after treatment.

Vascular smooth muscle cell migration assay

Migration assays were performed by using 24-well cell culture inserts with 8.0 μm pore size polyethylene terephthalate

membranes (Corning, NY, USA) of 10 mm in diameter. Cells were seeded into the transwell chamber at 1×10^5 cells/well and incubated with luteolin at the indicated concentrations. H_2O_2 1 μM was added to the lower chambers, which were filled with 600 μl DMEM. After 12 h of incubation at 37°C in 5% CO_2, non-migrated cells in the upper side of the membrane were removed with cotton swabs, and the cells on the lower surface of the membrane were fixed in 4% paraformaldehyde for 30 min and stained in hematoxylin and eosin solution for 15 min. The number of migrated cells were counted in five randomly selected squares per well with a light microscope under ×100 magnification and the control VSMC migration was indicated as 1 and results were expressed as relative migration rate.

Immunoblotting analysis

Cells were lysed with lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. Lysates were centrifuged at 12 000*g* at 4°C for 15 min. Equal amounts of the soluble protein were denatured in sodium dodecylsulfate (SDS), electrophoresed on an 11% SDS–polyacrylamide gel and transferred to a PVDF membrane. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG antibodies were used against respective primary antibody. The results were photographed after 5-bromo-4-chloro-3-indolyl phosphate/nitroblue-tetrazolium (BCIP/NBT) coloration. The results were evaluated by densitometry analysis.

Statistical analysis

All data were shown as mean rank. Statistical analysis was performed with Kruskal–Wallis test by SPSS 16.0 software. $P < 0.05$ was considered to be significant.

Results

Luteolin inhibits H_2O_2-induced proliferation of vascular smooth muscle cells

To determine the effect of luteolin on VSMC cytotoxicity or cell proliferation, VSMCs were pretreated with luteolin or PBS for 12 h, and then the MTT assay was performed as described in the Methods. The cell viability assay indicated that VSMCs' viability in the presence of 50 μM luteolin was greater than 99% and none of the luteolin concentrations used displayed significant cytotoxicity ($P > 0.05$) (Figure 2a). H_2O_2 plays a role in cell proliferation and the results indicated 1 μM H_2O_2 treatment significantly increased the proliferation

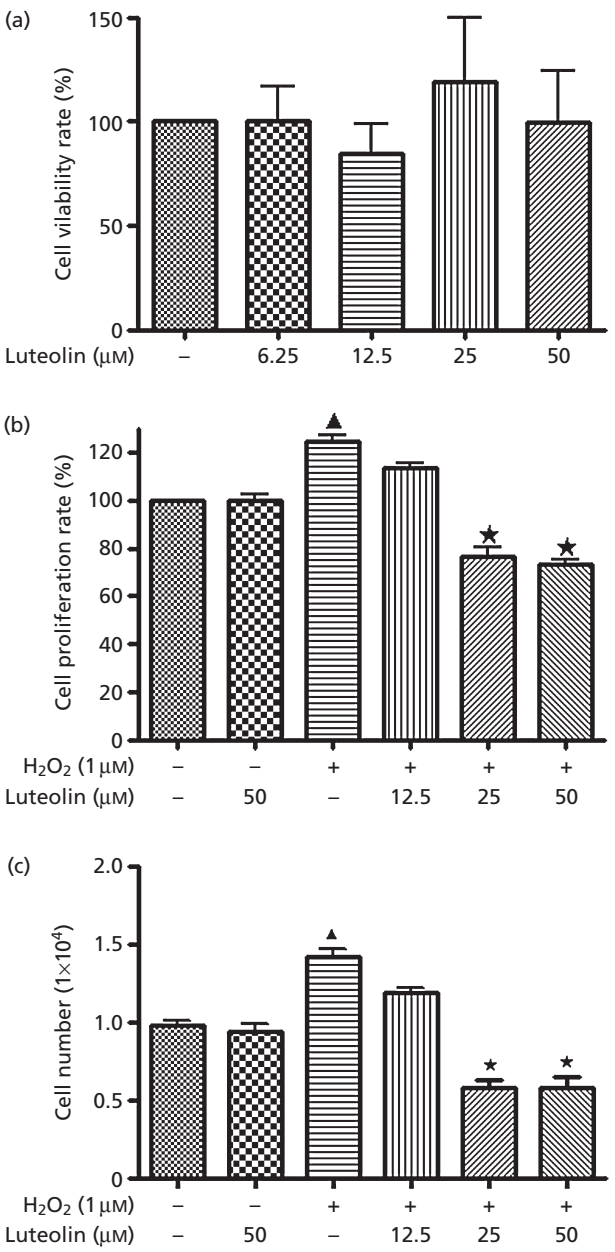

Figure 2 Effect of luteolin on VSMC toxicity and proliferation induced by H_2O_2. (a) VSMCs were cultured in 96-well plates and incubated with 0, 6.25, 12.5, 25 or 50 μM of luteolin for 48 h. Thereafter, the cell viability rate was determined by MTT assay as described in Material and Methods. (b) VSMCs were stimulated by 1 μM H_2O_2 after being pre-treated with indicated concentrations of luteolin for 12 h and then MTT assays were performed. Cell proliferation rate in the absence of treatment (control) was taken as 100%. (c) Cell proliferation was measured by cell counting. Data were obtained from three independent experiments and expressed as indicated. ▲$P < 0.001$, compared with the control; ☆$P < 0.001$, compared with the H_2O_2 group.

of growth-arrested VSMCs compared with the control group ($P < 0.001$). In the presence of 25 and 50 μM luteolin, 1 μM H_2O_2-stimulated cell proliferation was significantly inhibited ($P < 0.001$) after incubation with luteolin before the 12-h treatment with H_2O_2. Luteolin 50 μM can suppress the cell proliferation to the lowest level by about 50% (Figure 2b and 2c).

H_2O_2-stimulated migration of vascular smooth muscle cells was suppressed by luteolin

The effect of luteolin on the migration of VSMCs treated with H_2O_2 was measured by a migration assay. The migration of VSMCs was visibly promoted from the upper to the lower chamber with 1 μM H_2O_2 (Figure 3a). Incubation with H_2O_2 caused VSMCs to migrate to more than 200% over the control group ($P < 0.001$) (Figure 3b). In this study, the migration-promoting effect of H_2O_2 was significantly abrogated by pre-treatment with 25 μM or 50 μM luteolin ($P < 0.001$). Luteolin 50 μM completely suppressed VSMC migration to values roughly three times that of H_2O_2-stimulated cells.

H_2O_2-stimulated Src phosphorylation was suppressed by luteolin

Studies from other laboratories showed that Src kinase plays an important role in proliferation and migration.[24,28,29] Luteolin might play an antioxidant role by suppressing the activation of Src and subsequently blocking the production of ROS. To that end, we tested the effect of luteolin on H_2O_2-stimulated Src phosphorylation in VSMCs. Luteolin 50 μM visibly inhibited the activation of Src kinase induced by H_2O_2 (Figure 4).

Effect of luteolin on Akt activation by H_2O_2

To demonstrate whether luteolin inhibited H_2O_2-induced VSMC proliferation or migration by affecting the activation of Akt signals, we analysed the phosphorylation levels of PDK1, Akt (308) and Akt (473) in response to H_2O_2 after pre-treatment with luteolin for 12 h. The stimulatory effect of H_2O_2 on the Akt pathways in VSMCs was induced as early as 5 min and lasted up to 60 min (Figure 5). After H_2O_2 activation, both p-Akt (308) and p-Akt (473) reached their peak activating level ($P < 0.001$), before returning to the background levels. The results showed that H_2O_2-increased Akt

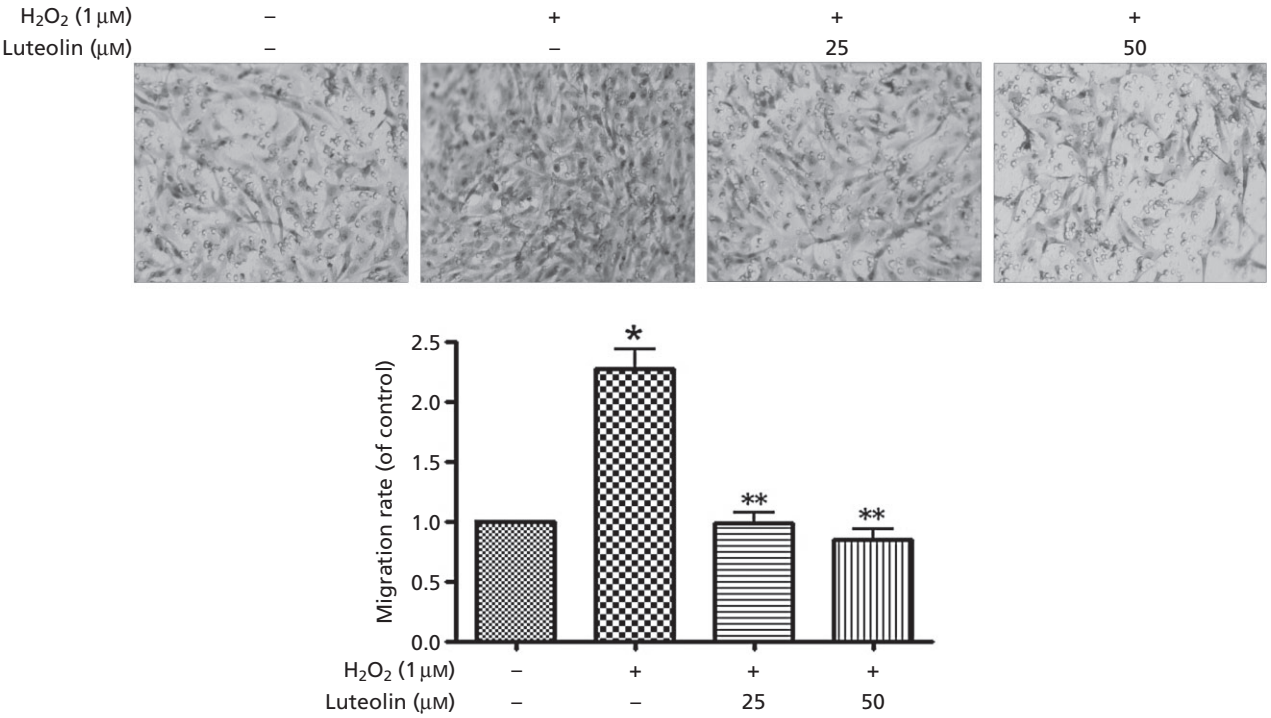

Figure 3 Treatment with luteolin dose-dependently reduced H_2O_2-stimulated VSMC motility in a transwell migration assay. VSMCs' migration rate was examined after H_2O_2 stimulation with or without various concentrations of luteolin. Bright-field images of randomly selected squares per group (×100). Luteolin attenuated the number of starved-VSMC migration in response to 1 μM H_2O_2 stimulation. The cells' migration rate in the absence of treatment was taken as 1. Experiments were performed in triplicate. *$P < 0.001$, compared with control; **$P < 0.001$compared with the H_2O_2 group.

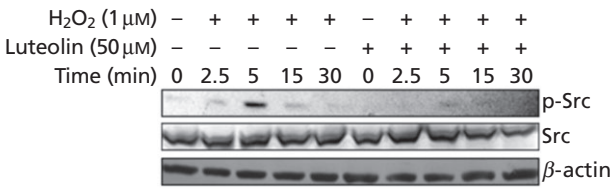

Figure 4 Luteolin inhibited activation of Src kinase elicited by H_2O_2. VSMCs were treated with or without 50 μM luteolin for 12 h in the presence or absence of 1 μM H_2O_2 for indicated time. Cells were lysed and immunoblotted with antibodies directed against p-Src and Src. Equal loading of protein was confirmed by *β*-actin. This is representative of one of three experiments with similar results.

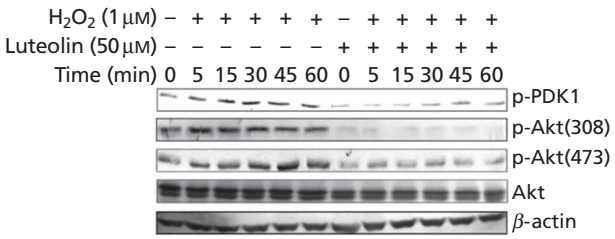

Figure 5 Luteolin significantly attenuated Akt and PDK1 phosphorylation induced by H_2O_2. After pre-treatment with 50 μM luteolin for 12 h VSMCs were stimulated by H_2O_2 (1 μM) for the time indicated. Cells were lysed and analysed using antibodies to p-PDK1, p-Akt(308), p-Akt(473) and Akt. Equal loading of protein was confirmed by *β*-actin. This is representative of one of three experiments having similar results.

phosphorylation was suppressed by luteolin ($P < 0.001$). Furthermore, we found that luteolin also had a remarkable impact on the phosphorylation level of PDK1, which is an upstream kinase of Akt.

Discussion

Much experimental evidence supports a cardiovascular protective role against atherosclerosis for the dietary flavonoids ubiquitously present in fruits and vegetables.[30] Epidemiological studies have suggested that the consumption of a flavonoid-rich diet is associated with a lower risk of cardiovascular disease. The proliferation and migration of VSMCs, as well as endothelial cell apoptosis, play an important role in the pathogenesis of atherosclerosis. The effect of luteolin on endothelial cells has been reported,[17] although few attempts have been made to test the function of luteolin on VSMC proliferation and migration. Other flavonoids, such as chrysoeriol, significantly inhibit platelet-derive growth factor (PDGF)-induced migration and proliferation of human aortic smooth muscle cells.[15] Pinkaew *et al*. proposes that morelloflavone blocks injury-induced neointimal hyperplasia via the inhibition of VSMC migration.[31]

The purpose of this study was to determine whether luteolin might affect the proliferation and migration of VSMCs and, if so, what molecular mechanism was involved in this effect. VSMC migration and proliferation are crucial events underlying the complications of vascular injury. This study proved that the proliferation and migration of VSMCs can be significantly induced by 1 μM H_2O_2. Our results indicated that 50 μM luteolin significantly suppressed the induced VSMC proliferation and migration without having any resulting toxic effect (Figures 2 and 3).

Luteolin is a type of flavonoid thought to possess an antioxidant and free radical scavenger function.[12] Studies have shown that ROS play a major role in vascular pathological changes that can promote cell proliferation and migration. Src is an important kinase that mediates oxidant stress in ROS pathways and is a very prompt responsive protein for tyrosine phosphorylation in H_2O_2-stimulated signalling events in VSMCs.[25,29] This study demonstrated that luteolin could exert its antioxidant effect by reducing the activation of Src and thus suppress the proliferation and migration of VSMCs.

Akt is activated after angioplasty or in the early stage of atherosclerosis and the Akt pathway is crucial for mediating cell survival, proliferation, migration and growth and early response gene expression. Fernandez-Hernando found that the absence of Akt reduces VSMC proliferation and migration.[21] Luteolin exerts its anti-cancer activity through suppressing the activation of Akt kinase.[32] Thus, we tested the hypothesis that luteolin could inhibit VSMC proliferation and migration using the same mechanism as it does in cancer cell lines. In a similar manner, we found that luteolin had the capacity to block H_2O_2-stimulated PDK1/Akt signalling pathways in VSMCs. Both H_2O_2-induced PDK1 and Akt phosphorylation significantly returned to the basal levels in VSMCs pre-treated with luteolin (50 μM) (Figure 5). Luteolin suppressed H_2O_2-induced proliferation and migration in VSMCs by attenuating the phosphorylation of PDK1, Akt (Thr308) and Akt (Ser473).

Akt can be phosphorylated by PDK1 and may also be activated by Src kinase. Src and PI3Ks are proximal components in migration signalling cascades.[26] Yamboliev showed that PI3K/Akt and Src are important enzymes in the spreading and migration of VSMCs.[13] It has been suggested that different signalling pathways regulate the proliferative and migratory response to H_2O_2 and these findings indicated that the effects of luteolin on VSMCs might be mediated by a coordinated inhibition of the Src and Akt signalling pathways.

Conclusions

In conclusion, developing an appropriate and effective medication that can inhibit proliferation and migration of VSMCs

has become an important goal in the prevention of atherosclerosis and restenosis after angioplasty. With the wide application of Chinese medicine in the cardiovascular system, the priority of most research has shifted to find a new, efficient and economic medication, with low toxicity, that can inhibit proliferation and migration of VSMCs. In this study, we observed that luteolin significantly suppressed the proliferation-inducing and migration-inducing effects of H_2O_2 on VSMCs through the Akt and Src signalling pathways. Based on the data presented here, luteolin may prove to be a potential therapeutic agent for the prevention and possible treatment of atherosclerosis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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