RESEARCH ARTICLE

Luteolin Suppresses Endothelial Permeability and Nitric Oxide Scavenging Effects

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Abstract

BACKGROUND: Numerous inflammatory diseases are linked to increased endothelial permeability through the nitric oxide (NO) flux in endothelial cells. Luteolin has *in vitro* and *in vivo* anti-inflammatory properties and has been reported to reduce endothelial permeability. However, the exact mechanism/s are yet to be determined. Thus, the present study was conducted to investigate the effects of luteolin in reducing endothelial permeability *in vitro* using bradykinin (BK) or sodium nitroprusside (SNP) through the NO pathway and the NO radical scavenging property of luteolin.
METHODS: Human umbilical vein endothelial cells (HUVECs) in the treatment groups were dosed with luteolin at 5, 10, and 25 µM concentrations and allowed to incubate for one hour prior to induction. The L-NAME or HOE 140 were administered prior to the induction of BK or SNP in HUVECs. The NO radical scavenging test, the nitrite determination assay using L-NAME as antagonist, and the *in vitro* vascular permeability testing using HOE 140 as antagonist were performed.
RESULTS: Endothelial permeability was decreased dose-dependently by 5, 10, and 25 µM luteolin *in vitro* via lowering NO generation. In comparison to HOE 140, luteolin suppressed the enhanced endothelial permeability more effectively. The suppression was 98.02% by 25 µM luteolin compared to HOE 140 94.05%. It was also discovered that luteolin, when incubated with SNP in a dose-dependent manner, possessed potent NO radical scavenging activities.
CONCLUSION: The current data demonstrated luteolin's ability to scavenge NO radicals and significantly decrease endothelial permeability through the NO route. Thus, in complementary medicine, luteolin might be potential to improve endothelial permeability suppressor in reducing inflammation.
KEYWORDS: luteolin, endothelial permeability, NO, scavenging property, HUVECs

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Introduction

Naturally occurring polyphenolic chemicals, flavonoids are significant secondary metabolites of plants that are vital in preventing oxidative damage. The health advantages of flavonoids are numerous in humans due to their great efficacy and comparatively low toxicity. Numerous flavonoids have been shown to offer significant adjuvant and preventative therapeutic benefits.(1) One of the many pharmacological characteristics that have been studied the most is their ability to reduce inflammation. Vasodilation is caused by an inflammatory process, and this is followed by an increase in endothelial permeability, which permits the passage of beneficial cells to the site of inflammation.(2) One of the primary indicators of inflammation is swelling, which is brought on by an increase in extravascular fluid at the site of inflammation due to the increased endothelial permeability. (3) Hence, a crucial part of the entire inflammatory process is played by the enhanced endothelial permeability.

Nitric oxide (NO) has been identified in various studies as an important bioregulatory molecule serving multiple functions like vasodilation and hypermeability of endothelial cells.(4) Numerous vascular disorders, including atherosclerosis and hypertension, are known to be caused by dysregulation of the endothelial NO synthase (eNOS), the primary source of NO synthesis in endothelial cells.(5) Stimuli like vascular endothelial growth factor (VEGF), shear stress, and various agonists like bradykinin (BK) induces phosphorylation of eNOS and thus inducing NO production. The increased NO concentration will then increase the endothelial permeability.(6) Many phytocompounds may reduce endothelial permeability. Phytocompounds especially flavonoids have been reported to exhibit many beneficial health effects. Quercetin, myricetin and kaempferol are commonly investigated.(1)

Luteolin, another flavonoid commonly used in Chinese traditional medicine commonly found in celery, parsley, onion leaves, carrots, peppers and apple skins (7), possesses various pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities through various preclinical studies (8). The mechanism of the potent anti- inflammatory properties of luteolin, both in vitro and in vivo, in modulating the immune system has not yet been fully understood.(3) The specific anti-inflammatory effects of luteolin can only be partly demonstrated by the antioxidant and free radical scavenging capacities of luteolin.(9) Since the exact mechanism of action of luteolin is unknown, it would be very helpful to ascertain how luteolin suppresses increased endothelial permeability to assess how successful it is as a substitute or prophylactic anti-inflammatory medication.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) (EndoGRO, MerckMillipore, Burlington, MA, USA) between passages 2 to 5 were used for the *in vitro* vascular permeability assay and the nitrite determination assay. Intact confluent monolayer cells were first grown before harvested for assays. Cascade Biologics Medium 200 Phenol Red-free cell culture medium (ThermoFisher Scientific, Waltham, MA, USA), which was enhanced with low serum growth supplement (LSGS) and penicillin-streptomycin antibiotics (ThermoFisher Scientific), was used to grow HUVECs until confluent before the detachment using trypsin (ThermoFisher Scientific). HUVECs were later divided into four main groups; the positive control, the negative control, the treatment group and the basal group.

in vitro Vascular Permeability Assay

The endothelial permeability of the HUVECs was determined with the *in vitro* Vascular Permeability Assay Kit (Cat. No. ECM640; Chemicon International, Tokyo, Japan) Protocols for the study were modified and optimized from the assay instructions provided by Chemicon International. Cells were incubated for 72 hours or until monolayer was formed.

For the treatment groups, luteolin (Cat. No. 10004161; Cayman Chemical, Ann Arbor, MI, USA) was added to reach 5, 10, or 25 µM of concentration. Meanwhile, for the positive control group, HOE 140 (Cat. No. B3259; Sigma-Aldrich, St. Louis, MO, USA) was added to reach final concentration of 10 µM. The plate was then incubated for 1 hour in the tissue culture incubator. HUVECs were then induced with 1 µM BK (Cat. No. B3259; Sigma-Aldrich) followed by the addition of FIT-C Dextran (Chemicon International). The plate was read at 485/530 (excitation/ emission) using fluorescence plate reader (ThermoFisher Scientific). The reading was directly proportional to the endothelial permeability of the HUVECs. The permeability index and the percentage of suppression were then calculated.

Nitrite Determination Assay

The determination of nitrite levels was conducted by using the Griess (Cat. No. G4410; Sigma-Aldrich).(10) Two separate tests had been conducted by using two different chemical inducers, namely BK and sodium nitroprusside (SNP) (Cat No. 567538; Calbiochem, San Diego, CA, USA) to determine the efficacy of luteolin in suppressing NO production in HUVECs with increased endothelial permeability.(11) The treatment groups were then pre-treated with 5, 10, 25 µM luteolin, while the positive control was pre-treated with the antagonist, 100 µM Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Cat. No. N5751; Sigma-Aldrich) for 1 hour. Then, 1 µM BK was introduced to all groups and Griess reagent was added into the well plate. The absorbance was read with a spectrophotometer at a wavelength of 540 nm. Similar test with 1 mM SNP as the inducer instead of BK had been carried out to reconfirm the capacity of luteolin in suppressing NO production. Similar steps were taken in conducting the entire test as discussed earlier except that BK was replaced by SNP. The readings referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

NO Radical Scavenging Test

The scavenging effect of luteolin on NO radicals was measured according to the previously described methods. (12) SNP was first dissolved in phosphate buffered saline (PBS). For the treatment groups, luteolin in final concentrations of 5, 10, and 25 µM were then added in the test tubes of 25 mM SNP solution. On the other hand, for the positive control group, 100 µM L-NAME was incubated with 25 mM SNP solution. The negative control group was given the standard 25 mM SNP solution. The tubes were then incubated at room temperature for 2 hours. The aliquot of the incubated solution was then removed from the test tubes and diluted with Griess reagent. The absorbance was immediately read at 570 nm.(13) The readings referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Statistical Analysis

The results were expressed as mean±standard error of mean (SEM) from three separate experiments and were analyzed statistically by one-way analysis of variance (ANOVA) and the Tukey's HSD test.

Results

Luteolin Reduced Vascular Permeability Index of BK-induced HUVEC Cells

There were no observed changes to the cells morphology during the experiments. The observed results of *in vitro* vascular permeability assay were shown in Table 1. The negative control was found to have a permeability index of 44.320±4.803%. When a comparison test was conducted between all groups and the negative control, the positive control (2.638±3.089%) showed a significant difference (p<0.001) from the negative control. The positive control significantly suppressed the increased permeability of the BK-induced HUVECs at a percentage of 94.05%.

There were significant differences (p<0.001) between all three treatment groups and the negative control. Luteolin at the dose of 5 µM and 10 µM had demonstrated a suppression of 69.27% and 87.16%, respectively. However, 25 µM luteolin had shown the highest suppression of the increased permeability of the BK-induced HUVECs at a percentage higher than the positive control (98.02%). Endothelial permeability was decreased dose-dependently by 5, 10, and 25 µM luteolin. In comparison to HOE 140, luteolin suppressed the enhanced endothelial permeability more effectively.

Luteolin Reduced Nitric Concentration of BK- and SNP-induced HUCEVs Cells

The results obtained from the nitrite determination assay of BK-induced cells were shown in Table 2. The basal cells were found to have a nitrite concentration of 5.203±0.1453 µM while the negative control had a concentration of 6.660±0.3686 µM. When a comparison test was conducted between all groups and the negative control, the positive control had shown a significant difference (p<0.01) from the negative control. The positive control had significantly suppressed the nitrite concentration of the BK-induced HUVECs at a percentage of 22.07%. Five µM luteolin was found to suppress the nitrite concentration of the BK-induced HUVECs in a dose dependent manner but at insignificant level (13.12%). However, there were significant differences (p<0.05) between the other two treatment groups (10 µM and 25 µM luteolin) and the negative control.

From the nitrite determination assay of the SNP-induced HUVEC cells, the results obtained were shown in Table 3. The basal cells were found to have a nitrite concentration of 5.563±0.1988 µM while the negative control had a concentration of 7.240±0.2030 µM. When a comparison test was conducted between all groups and the negative control, the positive control had shown a significant difference (p<0.01) from the negative control. The positive control had significantly suppressed the nitrite

Table 1. Luteolin in dose dependent manner reduced vascular permeability index of BK-induced HUVEC cells (n=3).

	Mean±SEM (%)	Suppression (%)
Negative Control	44.320±4.803[a]	-
Positive Control (10 µM HOE 140)	2.638±3.089[b]	94.05
5 µM Luteolin	13.620±3.763[b]	69.27
10 µM Luteolin	5.690±4.210[b]	87.16
25 µM Luteolin	0.8780±3.763[b]	98.02

[a-b]Mean with different superscript differ significantly at p<0.001.

Table 2. Luteolin in dose dependent manner reduced nitric concentration of BK-induced HUCEVs cells (n=3).

	Mean±SEM (μM)	Suppression (%)
Basal Level	5.203±0.1453[a]	-
Negative Control	6.660±0.3686[b]	-
Positive Control (100 μM L-NAME)	5.190±0.1353[a]	22.07
5 μM Luteolin	5.787±0.2302[b]	13.12
10 μM Luteolin	5.537±0.2087[a]	16.86
25 μM Luteolin	5.477±0.1674[a]	17.76

[a-b]Mean with different superscript differ significantly at $p<0.05$.

concentration of the SNP-induced HUVECs at a percentage of 18.74%. There were significant differences between all three treatment groups and the negative control. Five μM luteolin was found to suppress the nitrite concentration of the SNP-induced HUVECs at a significant level (74.72%, $p<0.01$). The reduction was in a dose dependent manner. The mean nitrite concentration of the SNP-induced HUVECs treated with 10 μM and 25 μM luteolin were found to have bigger suppresion percentage tahn the 5 μM luteolin group.

Luteolin Reduced NO Radical Scavenging Effects

From the NO radical scavenging test, the results obtained were shown in Table 4. The negative control (standard SNP solution) shows a nitrite concentration of 9.513±0.4822 μM. When a comparison test was conducted between all groups and the negative control, the positive control had shown a significant difference ($p<0.01$) from the negative control. The positive control had significantly suppressed the nitrite level of the SNP.

There were significant differences ($p<0.001$) between all three treatment groups and the negative control. Five μM luteolin was found to suppress the nitrite level of the SNP generated nitrite concentration at a significant level (36.16%). There were also highly significant differences ($p<0.001$) between the other two treatment groups (10 μM and 25 μM luteolin) and the negative control, with even higher suppression than the 5 μM luteolin.

Discussion

Phytocompounds have been proven to contain pharmacological activities as in modern drugs.(14-16) Many reports have been published regarding the benefits of flavonoids especially anti-inflammatory, anti-oxidative and anti-obesity.(17-18) Previous studies over recent years have demonstrated that luteolin possess potent anti-carcinogenic properties against different human cancer cell lines (19), antioxidant properties and anti-inflammatory properties as demonstrated previously (20). In this study, the anti-inflammatory property of luteolin was further evaluated in focus of its effect on the increased endothelial permeability, *i.e.*, the hallmark of inflammatory processes. The effect was carried out with three tests, namely the *in vitro* vascular permeability assay, the nitrite determination assay and the NO radical scavenging test.

As shown in this study, the luteolin compound has significantly suppressed the increased endothelial permeability in BK-induced HUVECs via the NO pathway. The reduction was in a dose dependent manner. The

Table 3. Luteolin in dose dependent manner reduced nitrite concentration of SNP-induced HUCEVs cells (n=3).

	Mean±SEM (μM)	Suppression (%)
Basal Level	5.563±0.1988[a]	-
Negative Control	7.240±0.2030[b]	-
Positive Control (100 μM L-NAME)	5.883±0.1584[a]	18.74
5 μM Luteolin	5.987±0.1517[a]	17.31
10 μM Luteolin	5.663±0.1648[a]	21.78
25 μM Luteolin	5.630±0.1537[a]	22.24

[a-b]Mean with different superscript differ significantly at $p<0.01$.

Table 4. Luteolin in dose dependent manner reduced the NO radical scavenging effects (n=3).

	Mean±SEM (µM)	Suppression (%) [Compared to L-NAME]
Negative Control	9.513±0.4822[a]	-
Positive Control (100 µM L-NAME)	6.593±0.2872[b]	-
Luteolin 5 µM	6.073±0.2367[b]	7.89
Luteolin 10 µM	5.897±0.2585[b]	10.56
Luteolin 25 µM	5.830±0.4115[b]	11.57

[a-b]Mean with different superscript differ significantly at $p<0.01$.

significant suppression confirms the anti-inflammatory properties of the compound making it an interesting compound for further studies before use as a preventive medicine. From the data analysis, the suppression of the increased endothelial permeability by luteolin is highly significant when compared to the effect of HOE 140, antagonist of BK (negative control). The percentage of suppression of luteolin at the dose of 25 µM, 98.02% has been found to be at a level higher than the positive control (94.05%). The study has also demonstrated that the effect of luteolin the increased endothelial permeability is dose dependent and it has been found that luteolin exhibits a higher suppression at a higher dose. The results of the *in vitro* vascular permeability assay test reveal the ability of luteolin in suppressing the increased endothelial permeability when BK was used as the inducer. HUVECs pre-treated with luteolin have significantly reduced the increased endothelial permeability.

In the present studies, the effect of luteolin in suppressing the NO concentration in HUVECs with increased endothelial permeability has been evaluated. Two different inducers have been used, *i.e.*, BK and SNP. Both inducers have been proven in previous studies to increase permeability of the endothelial cells through a similar pathway, *i.e.*, the Ca^{2+} dependent pathway.(6,21,22) Hence, the effect of luteolin in suppressing concentration of NO can be evaluated and confirmed through the two different inducers used in the study. We demonstrated that luteolin suppresses NO concentration in BK-induced HUVECs at all three doses tested. The effect is dose dependent as the suppression has been shown to increase with the dose applied. However, the significant effect of the suppression is only seen in BK-induced HUVECs treated with 10 and 25 µM luteolin. The data analysis has shown that the effect of suppression by luteolin at the dose of 5 µM to be insignificant when compared to the effect by L-NAME, the antagonist of NO production (negative control). This is probably due to the low concentration, causing luteolin failed to suppress the effect of BK on NO production in the HUVECs. The maximum suppression of the NO concentration in HUVECs induced with BK by luteolin is 17.76 % at the dose of 25 µM. Indeed, these results were as potent anti-inflammatory as other drugs.(23)

When the similar test is conducted with the second inducer, *i.e.*, SNP, luteolin has shown significant suppression of the NO concentration in the SNP-induced HUVECs at all three tested doses when compared to the negative control. The 10 µM and 25 µM luteolin have shown high percentage of inhibition higher than that of the negative control, *i.e.*, 21.78% and 22.24%, respectively. The results obtained to exhibit a dose-dependent trend. This confirms the ability of luteolin in suppressing the NO concentration of HUVECs with increased endothelial permeability. This marks a significant important finding that luteolin reduces increased endothelial permeability by targeting the NO concentration of the induced HUVECs. With the intervention with luteolin, it suppresses the concentration of NO, which will in turn suppress the production of cyclic guanosine monophosphate (cGMP) due to lack of NO radical. This will eventually lead to a reduced endothelial permeability. In a nutshell, the study has demonstrated that luteolin has a significant capacity in reducing the NO concentration of endothelial cells with increased endothelial permeability regardless of the inducer.

Compounds with NO radical scavenging property are an alternative NO synthase (NOS) inhibition and may be used to reduce toxic levels of NO without eliminating the low levels totally which may be beneficial. The present study found that luteolin possesses strong NO radical scavenging property. Results revealed all three tested doses of luteolin (5, 10, and 25 µM) have significantly high NO radical scavenging property. Percentage of suppression of the NO radical is high to a level higher than the positive control with the maximum percentage of 38.71% at the

dose of 25 µM. This marks the strong scavenging capacity of luteolin.

No general mechanism of the free radical-scavenging property of luteolin has been described. It was suggested that the 2, 3-double bond might be responsible for delocalizing electrons from the B ring giving the potential anti-oxidative and radical scavenging property to the flavonoids.(24) Hence, luteolin's strong NO radical scavenging property might be due to the 2,3–double bonds. This current study had limitation of direct disease related oxidative stress. Indeed, herbs are reported to be useful in obesity related oxidative stress.(25) Therefore, future studies of other possible pathways such as protein kinase C or suppression the increased concentration of intracellular calcium may lead to the suppression of increased endothelial permeability. However, further studies are required to determine the minimal effective doses and identify the pathway of action of luteolin upstream from NO. This is extremely crucial to evaluate the benefits of luteolin as an increased endothelial permeability suppressant in anti-inflammation as inflammation is a major etiology of many chronic diseases.(26)

Conclusion

The present study showed the significant ability of luteolin in scavenging NO radicals and decreasing endothelial permeability through the NO route. Thus, in complementary medicine, luteolin might be potential to improve endothelial permeability suppressor in reducing inflammation. However, further studies are needed to elucidate the exact molecular mechanism of the suppression of endothelial permeability by luteolin.

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Authors Contribution

ZA and MNH were involved in the conceptualization of the study. WTY, SSC, ZA developed the methodology of the study. ZA prepared the resources for the study. WTY and SSC perfomed the data collection and formal analysis. MSOF performed the statistical analysis. WTY, SSC, and MNH prepared the draft of the manuscript. MNH edited the manuscript during the reviewing process. All authors reviewed the results and approved the final version of the manuscript.

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