Evaluation of Anti-angiogenic Activities of Aqueous Extracts of Regular and Selenium-rich Green Teas Using Chick Chorioallantoic Membrane as an Experimental Model

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ABSTRACT
Angiogenesis, a process of developing new blood vessels, is essential in tumor progression and metastasis. It is well received that consumption of green tea (Camellia sinensi) can reduce risk of cancers to some extent. Here we used the chick chorioallantoic membrane (CAM) as a model system to study any anti-angiogenic effects generated by crude water extract of regular green tea (R-GTE) and selenium-rich green tea (Se-GTE). Comparing to the control CAM treated with only distilled water, the CAMs treated with the same volume of crude extracts of both R-GTE and Se-GTE had much less new blood vessels formed, indicating clear anti-angiogenic effects by the green teas. Se-GTE was superior to R-GTE and this superiority was attributed to the presence of higher level of organic selenium in comparison to the R-GTE. The anti-angiogenic properties of water extracts of both R-GTE and Se-GTE could be clues to the reduced risk of coronary heart diseases and cancers following consumption of green tea reported in numerous studies.

Keywords: antiangiogenic effect, chick chorioallantoic membrane (CAM), regular green tea, selenium-rich green tea.

INTRODUCTION
Angiogenesis describes a process of new blood vessel development from the existing microvascular bed. Angiogenesis is essential for the embryonic development, regulation of the processes that occur during the female reproductive cycle (formation of corpus luteum and placenta) and in wound healing. It also plays a prominent role in the progression of several pathologic conditions such as arthritis, diabetic retinopathy, psoriasis, and tumor growth [1]. It is well established that neovascularization promotes the progression of tumors [2]. It has been reported that the angiogenic process is controlled by two major proangiogenic factors, matrix metalloproteinases (MMPs), which degrade extracellular matrices, and vascular endothelial
growth factor (VEGF), which strongly stimulates endothelial cells migration and proliferation and the formation of new blood vessels [3].

Chick chorioallantoic membrane (CAM) has been used as a model system for studying cancer behavior [4], properties of biomaterials [5], angiogenesis [3, 6], and photodynamic therapy [7]. Leng et al. [6] proposed several new applications for CAM in the study of retina and its vasculature with respect to microsurgical interventions. Something about cancer to support what you mentioned in the Abstract and Discussion?

It has been well established that regular consumption of green tea (R-GTE) can reduce the risk of coronary heart disease and cancer [8-11]. The beneficial effect on coronary diseases might be attributable, in part, to their ability to retard the progression of early atherosclerotic lesions [12, 13]. Maiti et al. [14] tested the effect of green tea polyphenols on angiogenesis using the chicken chorioallantoic membrane (CAM) assay and their results showed that green tea components were capable of reducing the vascularization on CAM that is induced by the angiogenin-like protein.

On the other hand, selenium-rich green tea (Se-GTE) showed higher antioxidant and prebiotic activities than R-GTE [15, 16] and this superiority may be due to the difference in the level of organic selenium. Although the antiangiogenic properties of green tea have been investigated previously, no studies, as far as we know, have investigated the effect of selenium-rich green tea on angiogenesis using the CAM assay. Accordingly, the aim of the present study was to compare between the regular green tea (with very low concentration of organic selenium) and selenium-rich (contains moderate level of organic selenium) regarding their impact on angiogenesis process using CAM assay.

MATERIALS AND METHODS

Preparation of tea extracts
Regular green tea (R-GTE) was purchased from retail shops while selenium rich green tea (Se-GTE) was obtained directly from China. The extracts were made by adding 100 mL water (100°C) to 1 g (1%) or 2 g (2%) tea leaves, brewing for 10 min with stirring and then removing solid matter by filtration. The filtrate was used on the same day of preparation.

Evaluation of anti-angiogenic properties of green tea extracts
A series of experiments were conducted to evaluate the anti-angiogenesis effects of extracts from two types of green tea, regular green tea (R-GTE) and selenium-rich green tea (Se-GTE), using Chicken Chorioallantoic Membrane (CAM) assay as described previously [17] with some modifications. Briefly, fertilized chicken eggs were purchase locally from Golden Coast Commercial, New Zealand and their surfaces were disinfected with 70% alcohol. The eggs were incubated in an incubator (R. COM Digital incubator, 20-PRO, United Kingdom) for 5 days at 37°C with fully humidified atmosphere. On the fifth day of incubation, the eggs were arranged vertically before cutting the egg shell. The egg shell was cut open from the wide end, above the air sac, and the inner membrane was left intact. The airspace can be visualized by holding the egg under intense light [6]. Thirty microliter aliquots of tea extracts were placed directly on the membrane and then the opening was sealed with sterile cellophane tape and three eggs were used for each concentration. For the control CAM, distilled water was used instead of tea extracts. The eggs were incubated for 2 more days using the same incubator. On day seven, the membrane was removed and angiogenesis was photographically monitored (Olympus, Japan). The density of blood vessel formation in each treatment was assessed by comparing with that of a control CAM.

RESULTS AND DISCUSSION
The results of the present study suggested that both R-GT and SE-GT water extracts have an anti-angiogenic effect. Using different Experimental studies using different tumour angiogenic models, have shown that green tea extract and/or its polyphenolic substances such as catechins have strong anti-angiogenic effects [18-21]. Although the mechanism of the anti-angiogenic activity was not investigated in this study, some previous studies have shown that it is to be associated with inhibitory effects on VEGF production and VEGF receptor activity [22-26].

In a recent review, Rashidi et al. [27] reported that the polyphenolic compounds, especially Epigallocatechin-3-gallate (EGCG), from green tea are able to change the microRNAs (miRNA) expression profile associated with angiogenesis in various cancer types and also able to inhibit the vascular endothelial growth factors (VEGF) family.

The local application of water extracts from both regular green tea (R-GTE; Figure 2) and selenium-rich green tea (Se-GTE; Figure 3) to the chick embryo chorioallantoic membrane (CAM) strongly inhibited angiogenesis as indicated by a marked reduction in the number and length of small blood vessels after a 48-h incubation period. Figure 1 shows the normal growth of blood vessels which gets reduced/inhibited due to the presence of the water extracts from both R-GTE and Se-GTE as indicated in Figures 2 and 3, respectively. The blood vessels under green tea extracts were found to be thinner and less branching (Figures 2 and 3) when compared with the control CAM treated with distilled water only (Figure 1), indicating that an inhibitory effect is induced by the presence of tea water extracts. Our results concerning the antiangiogenic effect of regular green tea are in line with the results of the previous studies [16]. The superiority of Se-rich green tea over the regular green tea may be explained by the higher antioxidant activity of the Se-GTE in comparison to the R-GTE [14] and the higher concentration of the organic selenium [14, 15].

Although the mechanism of antiangiogenic properties of green tea is not investigated in the present study, it has been reported that drinking of green tea (GTE) or GTE-derived natural polyphenols exhibits antiangiogenic properties that may be explained by their ability to inhibit several key events of the angiogenic process such as proliferation and migration of endothelial cells, and through down-regulation of several angiogenic molecules, such as vascular endothelial growth factor (VEGF) and metalloproteinases (MMPs) [3, 28]. It has been reported that green tea polyphenols (GTPs) and epigallocatechin-3-gallate (EGCG) have the ability to decrease VEGF production in head and breast carcinoma cells by inhibiting epidermal growth factor receptor-related pathways of signal transduction [29, 30]. Another study found that (-)-epigallocatechin gallate (EGCG) suppressed the formation of new blood vessels [31]. Tange et al. [32] investigated the inhibitory effect of green tea catechins on ephrin-A1-mediated tumor angiogenesis and found that the green tea catechin EGCG inhibited ephrin-A1-mediated endothelial cell migration, as well as tumor angiogenesis, in a dose-dependent manner and suggested a novel antiangiogenesis application of EGCG in cancer chemoprevention. Another report mentioned that green tea appears to be emerging as a very important chemopreventive agent against cancer by virtue of its content of EGCG [33]. Moreover, it has been reported that drinking tea significantly prevented VEGF-induced corneal neovascularization in a mouse model [28].

In addition to EGCG and other catechins found in the regular green tea, Se-GTE contains organic selenium [14, 15]. Selenium is an essential dietary component for animals including humans.
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Accumulated evidence from prospective studies, intervention trials, and studies on animal models of cancer have suggested a strong inverse correlation between selenium intake and cancer incidence [34, 37]. Selenium appears to have a protective effect at various stages of carcinogenesis including both the early and later stages of cancer progression and several mechanisms for selenium-anticancer action have been proposed such as antioxidant protection, enhanced carcinogen detoxification, enhanced immune surveillance, modulation of cell proliferation (cell cycle and apoptosis), inhibition of tumor cell invasion and inhibition of angiogenesis [38].

CONCLUSIONS
In conclusion, the CAM is a convenient experimental model for angiogenesis/antiangiogenesis studies and the antiangiogenic properties of water extracts of both R-GTE and Se-GTE may explain the reduced risk of coronary heart diseases and cancer mortality following consumption of green tea shown by many previously published works. In addition, the Se-rich GTE offers a good combination of polyphenols and organic selenium, which are both have antioxidant and anti-cancer activities.

CONFLICT OF INTEREST STATEMENT
The Authors declare no conflict of interest.

REFERENCES

**Figure 1.** Chick chorioallantoic membrane (CAM). Photograph of mature chick CAM showing the dense network of blood vessels (control CAM).

**Figure 2.** Anti-angiogenic effects of water extracts from regular green tea on the chick embryo CAM. The local application of 30 microliters of 1% (panels 1 and 2) and 2% (panels 3 and 4) tea extract.
extracts to the CAM strongly inhibited the formation of new blood vessels as compared with the control CAM after a 48-h incubation period.

**Figure 3.** Anti-angiogenic effects of water extracts from selenium-rich green teas on the chick embryo CAM. The local application of 30 microliters of 1% (panels 1 and 2) and 2% (panels 3 and 4) tea extracts to the CAM strongly inhibited the formation of new blood vessels as compared with the control CAM after a 48-h incubation period.