

The preventive effects of G115 on balloon injury-induced neointima formation in rats

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Abstract

After percutaneous transluminal coronary angioplasty (PTCA), 30–50% of the patients may present with restenosis within 6 months. The aim of this study was to search for a preventive remedy against the balloon injury-induced neointima formation. Ginseng, with its wide indications on immune and cardiovascular functions, has prompted us to explore its role in neointima formation. In the present study, we aimed to explore if a standardized *Panax Ginseng* extract G115 was able to inhibit neointimal formation. With BrdU luminence assay, maximal proliferation of rat smooth muscle cells was reduced to 24% of control values by G115. Norepinephrine-induced vasoconstriction was antagonized in 21% and 44% by 1.44mg/ml and 2.88mg/ml of G115, respectively. Neointima-to-lumen area ratio of balloon-injured rat carotid arteries was reduced 77.3% by G115 as compared to the sham control. These results demonstrate the preventive effects of ginsenosides on angioplasty-mediated neointima formation. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Angioplasty; Restenosis, *Panax Ginseng*; Ginsenosides; G115

Introduction

Coronary heart disease is rated as one of the leading causes of death worldwide. Percutaneous Transluminal Coronary Angioplasty (PTCA) has been extensively applied as a clinical approach to treat coronary heart disease. However, the injury denudes the vascular endothelial cells and traumatizes the media. This damage results in acute loss of vasodilation capabilities, acute thrombosis, augmented platelet and leucocyte activation and adhesion [1–3].

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Consequently, denudation of endothelial cells may activate multiple signal transduction pathways in media ultimately resulting in proliferation and migration of smooth muscle cells [1–5]. This late intimal formation and arterial remodeling have been considered as one of the mechanisms leading to restenosis [6].

Restenosis is the angiographic narrowing at the angioplasty site in 30–50% of patients within 6 months of PTCA without stenting of the arteries, frequently accompanied by recurrence of angina symptoms [7]. Therefore, even though PTCA has been proven to be an effective therapy for saving people from myocardial infarction, restenosis has limited the benefit of angioplasty in clinical application. In many animal studies, it was found that gene therapy in dilating vessels or regulating NO synthesis was effective in preventing restenosis [8,9]. However, gene therapy is still away from perfection due to unsatisfied gene transferring efficiency and cytotoxicity. We therefore aimed to search for a pharmacological agent in preventing abnormal cell proliferation as well as inducing vascular relaxation to suppress balloon injury-induced restenosis.

Panax Ginseng has long been used in Chinese since Han Dynasty about 2000 years ago. Its main indications are to enhance stamina and capacity to cope with fatigue and physical stress [10]. The major active principles of *Panax Ginsen* are ginsenosides, the derivatives of the triterpene dammarane structure [11,12]. Minor components are amino acids, peptides, and minerals. There have been about 20 different ginsenosides extracted from roots, leaves, and flower buds of ginseng [11]. Aglycones of the common ginsenosides are 20(S)-protopanaxadiol (Rb1, Rb2, Rc, and Rd) or 20(S)-protopanaxatriol (Re, Rf, Rg1, and Rg2) structures, whose nomenclatures derive from the mobility of the ginsenosides in a one-dimensional thin-layer chromatographic system [13–15]. Various sugar moieties are found present in *Panax Ginseng*, including glucose, maltose, fructose, and saccharose. Conventional HPLC, as well as electro-spray HPLC and mass spectrometry are now available to quantitate and purify ginsenosides [14–16].

Activity analysis of ginseng revealed that the ginsenoside content depends on the species of ginseng, the manner of sample preparation, and the age and part of the plant extracted. The current available ginseng preparations may therefore differ greatly in pharmacological aspects. To provide the public with reliable information on ginseng preparations, American Botanical Council has analyzed several hundred of ginseng products using standard HPLC techniques. These results have encouraged the publics and research groups to use standardized ginseng to gain consistent effects. G115, a specific ginseng preparation used in many studies, is standardized to contain 4% ginsenosides [13,17].

It is generally believed that the injury-induced restenosis is partly resulted from the uncontrolled smooth muscle cell proliferation and vascular contraction due to endothelial dysfunction [18]. Souza et al. [19] indicate that excessive free radical generation early after arterial balloon injury may also account for restenosis. This is supported by the finding of Libby and Ganz [20] that reactive oxygen species enhance transcription of nuclear factor (κ) B in balloon-injured cells resulting in smooth-muscle-cell migration, replication, and accumulation and remodeling of extracellular matrix. Ginsenosides has been reported to prevent proliferation of vascular smooth muscle cells from rats [21]. A similar proliferation-inhibitory activity was also found in mesangial cell cultures by ginsenosides [22]. In addition to the anti-proliferative effects, ginsenosides are also found to enhance acetylcholine-induced vas-

cular relaxation [G23]. Due to the extensive effects of ginsenoside on cell proliferation and vascular relaxation, it is then feasible to propose that G115 may play a critical role in preventing balloon injury-induced restenosis. In order to assess the preventive effects of standardized ginseng G115 against restenosis, *in vitro* smooth muscle cell proliferation, *ex vivo* vascular relaxation, and *in vivo* neointimal formation affected by G115 were evaluated in the present study.

Materials and methods

Cell culture

The smooth muscle cells harvested from rat thoracic aorta were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Gibco BRL, Life Technologies), 2mM L-glutamine, 100 units of penicillin and 100mg of streptomycin per ml, and 1 mM sodium pyruvate. Cells were kept in a humidified 5% CO₂-95% air incubator at 37°C till 80% confluence before addition of G115.

Quantitative determination of ginsenoside Rb2 in G115

A Thermo Separation Products HPLC system consisting of a ConstaMetric 4100 solvent delivery system, a SpectraSYSTEM AS 3000 auto-sampler, a SpectraSYSTEM UV 2000 detector, and a SpectraSYSTEM software PC-1000 was used in this analysis. A gradient elution with two solvent systems, acetonitrile (A) and water (B) were performed. The program was run from 5% A to 60% A in 30 min and then changed back to 5% in another 5 min. The column was re-equilibrated at initial condition for 10 min prior to the next injection. The flow rate was set at 1.5 ml/min during the whole process. Analytical column was Merck Lichrospher 100 RP-18 (125 × 4.0 mm i.d., 5 μm). The elution was monitored at 203 nm and the sample injection volume was 10 μl. A calibration curve plotting was performed as follows: 250 ppm of standard Rb2 was diluted to a series of concentrations (125, 62.5, 50, and 25 ppm). The above standard solutions with five concentration levels were then individually injected to HPLC. A calibration curve was plotted based on the peak height versus concentration of standard. The ginsenoside Rb2 content in G115 was calculated according to the above calibration curve. Injection of extract sample was carried out in triplicate.

Chemiluminescence analysis of ginsenosides on smooth muscle cell proliferation

BrdU incorporation using chemiluminescence immunoassay was employed to assay DNA synthesis affected by G115. This non-radioactive method was an alternative approach to the [³H]-thymidine incorporation assay, providing a reliable and sensitive measurement of cellular proliferation. According to the manufacturer's protocol (Boehringer Mannheim, German), cells were incubated in the presence of 10 μg/ml, 100 μg/ml or 1mg/ml of G115 for 20 hours at 37°C. Fifty μl of BrdU /10⁶ cells were added to cells for additional four hours at 37°C. Dead cells were pre-washed away prior to trypsin dissociation. Equal amount of viable cells (10⁶ cells) in each culture well determined by Trypan blue exclusion was then fixed with FixDenat for 30 min followed by anti-BrdU incubation for 90 min at 25°C. Peroxidases conjugated to anti-BrdU were used to oxidize luminol in the presence of hydrogen peroxide. A reaction product in an excited state was formed followed by decaying to the ground state by

emitting light. The signal (photons) generated in enzyme-catalyzed light emitting reactions (luminescence) proportion to the DNA synthesis was then measured in OPTOCOMP I luminometer (MGM Instruments, Inc. Hamden CT, USA).

Ex vivo assay of G115 on contractions of aortic strips

Sprague Dawley (SD) rats (n=40) weighed 200–250g were sacrificed by spinal dislocation, the thoracic aorta was removed and placed in physiological salt solution (PSS in mM: 119 NaCl, 24.9 NaHCO₃, 11 D-glucose, 1.2 KH₂PO₄, 4.6 KCl, 1.2 MgSO₄·7H₂O, 1.5 CaCl₂). The adherent connective tissues were trimmed and the aortas were cut spirally (approximately 2 × 10mm). Sixteen aortic strips were subject to endothelial denudation by gentle touch with cotton swabs. All 40 aortic strips were then mounted in 5 ml tissue bath containing Krebs buffer of the following composition (in mM: 117 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.8 MgSO₄, 1.18 KH₂PO₄, 25 NaHCO₃, 11 dextrose, 0.03 EDTA) at 37°C and aerated with 95% O₂ and 5% CO₂. These aortic strips were divided into 5 groups: First, the norepinephrine group; Second, endothelial denudation with norepinephrine group; Third, norepinephrine plus G115 group; Fourth, endothelial denudation with norepinephrine plus G115 group; Fifth, L-NAME pretreatment followed by norepinephrine plus G115 group. Tension was recorded on Gould 2600s recorder via Grass Fore displacement transducer. An initial 1g loading tension was applied to each preparation and maintained throughout a 60–90 min equilibration period. N^G-Nitro-L-Arginine Methyl Ester (L-NAME, Research Biochemicals Incorporated, Natick, MA USA), a nitric oxide synthase inhibitor, was added to the fifth group 20 minutes prior to NE treatment at the concentration of 10⁻⁷M. Thereafter a fixed concentration of norepinephrine (10⁻⁷M) was used to induce aortic contraction. Upon the maximal contraction of aorta has been induced, 0.18mg/ml, 0.36 mg/ml, 0.72 mg/ml, 1.44 mg/ml, and 2.88 mg/ml of G115 were administered to the tissue bath with 5 min interval. PSS was added in the same volume of G115 to the First group as a negative control. The vascular relaxing effects of G115 can then be measured as compared to the PSS control.

In vivo assay of G115 on balloon injury-induced restenosis

SD rats weighted 300–400g were pretreated with low dose (2mg/kg) and high dose (200mg/kg) of G115 orally for 7 days followed by balloon injury. There were 6 rats in each group. Drugs were continued for 14 days after balloon injury to carotid arteries. At the time of surgery, rats were anesthetized using 10:1 Ketamine and Xylazine and the left site of common artery were exposed for balloon catheter insertion. After locating the bifurcation of external and internal carotid arteries, the internal carotid artery was temporarily ligated. The cranial side of external artery was permanently ligated and the region close to bifurcation was nicked and inserted with a balloon catheter. Until the tip of balloon catheter reached the common carotid artery, 1.5 atm pressure was applied to inflate the balloon. The inflated balloon was then moved back and forth 3 times to injure the vascular endothelium. Two weeks after balloon injury, rats were anesthetized with pentobarbital and perfusion-fixed with paraformaldehyde to minimize the possible morphologic changes of common carotid arteries. The contralateral arteries without balloon injury were also checked for the morphologic changes. Tissue sectioning was performed at the desktop microtome with 10 μm thickness. The paraffin embedded sections were processed for α-actin immunostaining to identify the smooth mus-

cle cells in the neointimal layer. After dewaxing formalin-fixed, paraffin-embedded tissue sections, the antigens were retrieved by heating in 10 mM citric acid buffer, pH 6 with 700 watt microwave for 2–3 min. Anti- α -actin was then applied to sections followed by anti-rabbit-HRP (Horse reddish peroxidase) hybridization. Sections were further processed for antigen visualization by reacting with DAB (diaminobenzidine) (Sigma, USA). HRP reacted with DAB to form a very stable, brown end-product at the site of the target antigen. After additional staining with hematoxylin and eosin, the morphological analysis of the sections was then processed by means of digital computing system (Sakoi NTSC Inc., Japan). The computer program Matrox Inspector (Matrox Electronic Systems Ltd., USA) were used to measure the neointimal thickness of the vessel wall.

Statistics

Data are expressed as mean \pm SEM. ANOVA was performed for statistical analysis of continuous variables followed by Newman-Keuls test. A p value <0.05 was considered statistically significant.

Results

Quantitative analysis of the ginsenoside content in G115

G115 was known as a standardized ginsenoside mixture. HPLC chromatograms of Rb2 standard and G115 were shown in Figure 1. The peak appeared at 11.894 min in Figure 1A

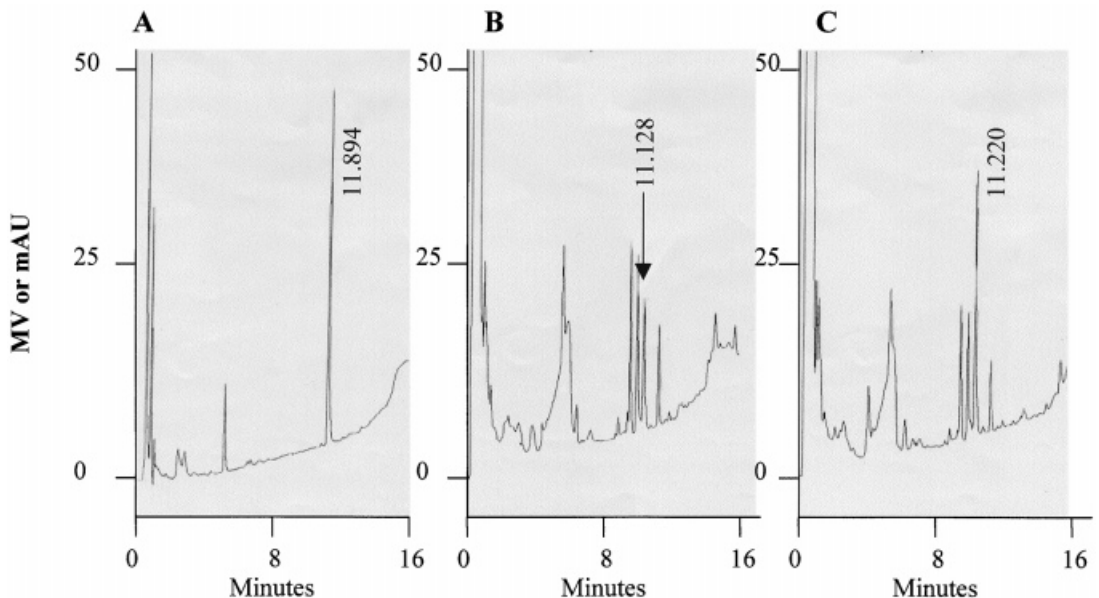


Fig. 1. HPLC chromatograms demonstrating the quantitative analysis of Rb2 content in G115. A: Injection of Rb2 standard showed a peak at 11.894 min. B: Measurement of G115 showed a peak at 11.128 min demonstrating the possible content of Rb2. C: Identification of Rb2 in G115 by injection of a mixture containing G115 and Rb2 standard.

was tentatively determined to be Rb2 because it was the base peak in this chromatogram. The corresponding peak showed in Figure 1B was at 11.128 min. This identification was confirmed by injection of a mixture containing G115 and Rb2 standard (Figure 1C). Five concentrations of Rb2 (25, 50, 62.5, 125, 250 ppm) subjected to HPLC analysis resulted in peak height of 4325, 9685, 12157, 23347, 41815 RU (relative units), respectively. A standard curve was then constructed accordingly. The contents of ginsenoside Rb2 in G115 was determined based on this curve to be 86.46 ± 1.30 ppm or 84.46 ± 1.30 $\mu\text{g/ml}$ ($n=3$).

Chemiluminescence analysis of ginsenosides on smooth muscle cell proliferation

G115 was first tested *in vitro* for its antiproliferative effects on culture smooth muscle cells prior to its *in vivo* analysis. BrdU incorporation using chemiluminescence immunoassay was a sensitive and alternative approach to the [^3H]-thymidine incorporation assay in assaying DNA synthesis. Cells were stimulated with 15% serum in the presence of 10 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, and 1mg/ml of G115 for 20 hours at 37°C followed by 4 hours of BrdU pulse incubation. Cells with more BrdU incorporation into DNA emitted stronger luminescence upon reacting with luminol and 4-iodophenol substrates. While 10 $\mu\text{g/ml}$ of G115 did not demonstrate a significant inhibition on cell proliferation, the maximal intensity of luminescence caused by 15% serum induction was significantly reduced to 64% and 76% of control by 100 $\mu\text{g/ml}$ and 1mg/ml of G115, respectively (Figure 2, $n=5$, $p < 0.05$).

Antagonism of G115 against the inotropic effects of norepinephrine on aortic strip

To determine if the preventive effects of G115 on balloon injury-induced stenosis are mediated by vasodilation, spirally cut strips of rat thoracic aorta were subjected to co-incubation

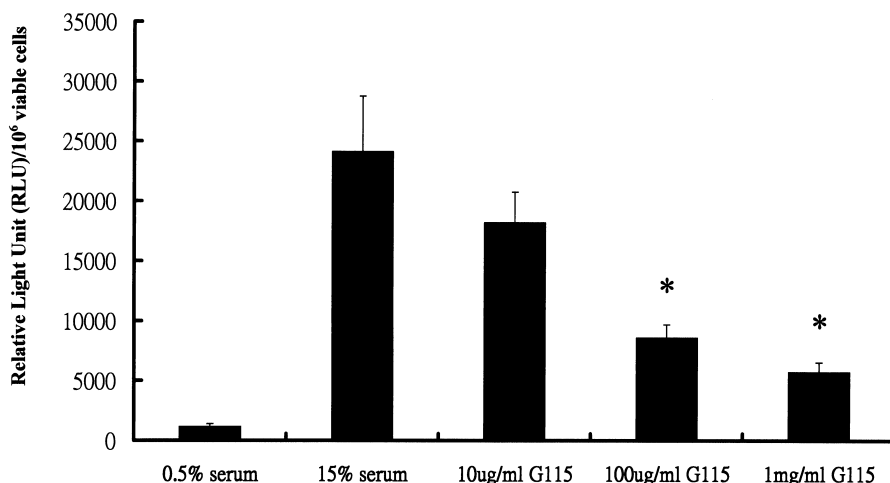


Fig. 2. Chemiluminescence assays of BrdU incorporation under the effects of G115 on culture smooth muscle cells. The 15% serum stimulated-BrdU incorporation expressed as relative light units (RLU)/10⁶ viable cells was significantly reduced by 100 $\mu\text{g/ml}$ and 1mg/ml of G115. Data are mean \pm SEM of 5 separate experiments. * indicates $p < 0.05$ as compared to 15% serum control.

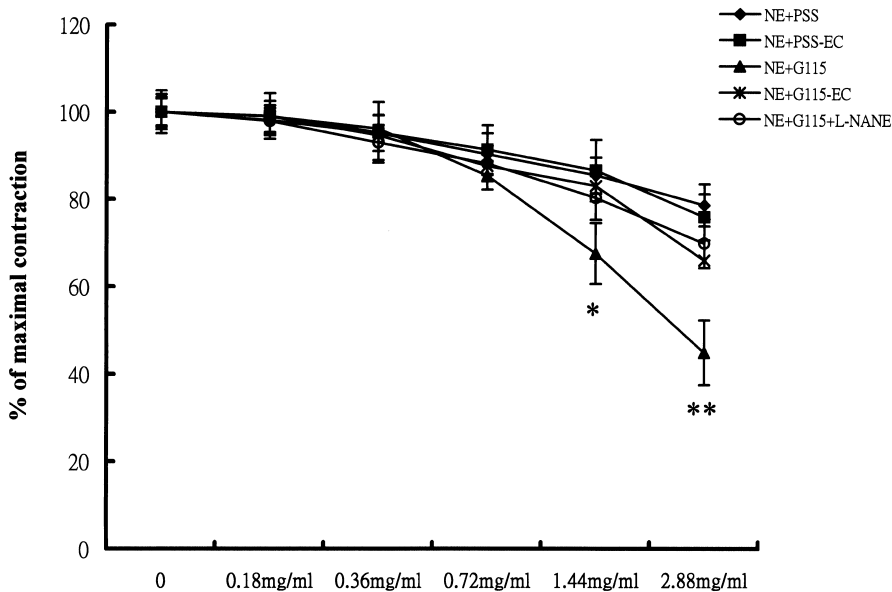


Fig. 3. Effects of G115 on norepinephrine-induced vasoconstriction in isolated rat aorta. Aortic strips were divided into 5 groups: NE+PSS, norepinephrine plus physiological salt solution; NE+PSS-EC, endothelial denudation with norepinephrine plus physiological salt solution; NE+G115, norepinephrine plus G115; NE+G115-EC, endothelial denudation with norepinephrine plus G115; NE+G115+L-NAME, L-NAME pretreatment followed by norepinephrine plus G115. Data are mean \pm SEM of 8 separate experiments. * $p < 0.05$; ** $p < 0.01$ as compared to NE + PSS controls.

of norepinephrine and G115. G115 reduced the norepinephrine-induced vasoconstriction in a dose-dependent manner. At concentrations lower than 0.72 mg/ml, G115 showed no inhibition on the inotropic effects of norepinephrine (10^{-7} M). While the doses of G115 increased to 1.44 and 2.88 mg/ml, the percentage of maximal contraction in reference to the PSS control was reduced by 21% and 44%, respectively (Figure 3, $n=8$, $p<0.05$). Such vascular relaxing effects of G115 were reversed when vessels were denuded on endothelium or pretreated with NO synthase inhibitor, L-NAME (Figure 3).

In vivo assay of ginsenosides on balloon injury-induced restenosis

Rats pretreated with low dose (2mg/kg) and high dose (200mg/kg) of G115 were killed two weeks after balloon injury. The carotid arteries were isolated and sectioned for hematoxylin and eosin staining. As shown in figure 4, panel A demonstrated the normal carotid artery without neointima formation. There was a significant neointima formation over the medial layer of the balloon-injured vessel (Figure 4B). The thickness of the neointimal layer was significantly reduced by either low or high dose of G115 (Figure 4C and 4D). The intact contralateral artery without balloon injury in the G115 treatment groups had no significant morphologic changes as compared to that of the sham controls. Using computer-assisted image analysis, the area ratio of neointima to media was compared between different groups. Figure 5 showed an area ratio of 2.16 for the balloon-injured vessels. This high ratio was significantly reduced to 0.49 and 0.46 in low dose and high dose of G115-treated vessels, respectively.

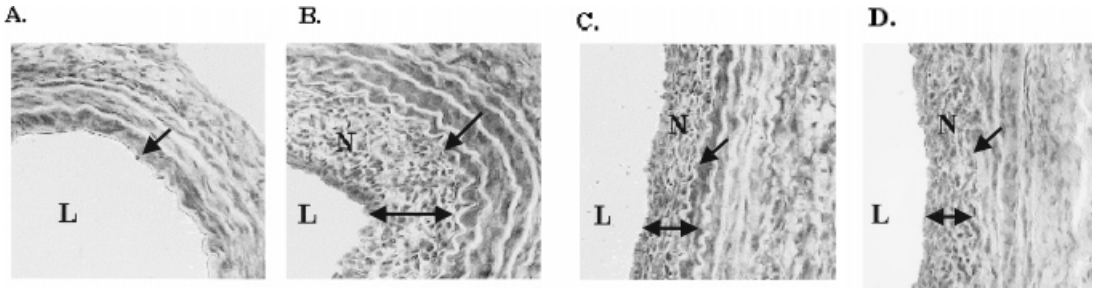


Fig. 4. The inhibitory effects of Ginseng Extracts on balloon injury-induced neointimal formation in rat carotid arteries. Smooth muscle cells present in the neointimal layer were identified with α -actin immunostaining. A: Normal vessel without neointimal formation. B: Vessel received balloon injury without ginseng treatment showed a significant neointimal formation as indicated by the double-arrow heads. C: Vessel pretreated with 2mg/kg G115 and D: Vessel pretreated with 20mg/kg G115 followed by balloon injury showed a significant reduction in neointimal formation. Single-arrow heads indicate the internal elastic lamina over which the neointima is formed. L:lumen; N: neointimal layer. Magnification: 400 \times .

Discussion

The primary objective of this study was to explore an herbal medicine in alleviating PTCA-mediated restenosis. With its wide applications in Chinese medicine, ginseng was deemed to be one of the potential candidates in preventing balloon injury-induced restenosis. The preventive concept as the essence of Chinese medicine has been drawing much attention in the Western society in these years. For example, in a recent issue of Biochemical Pharma-

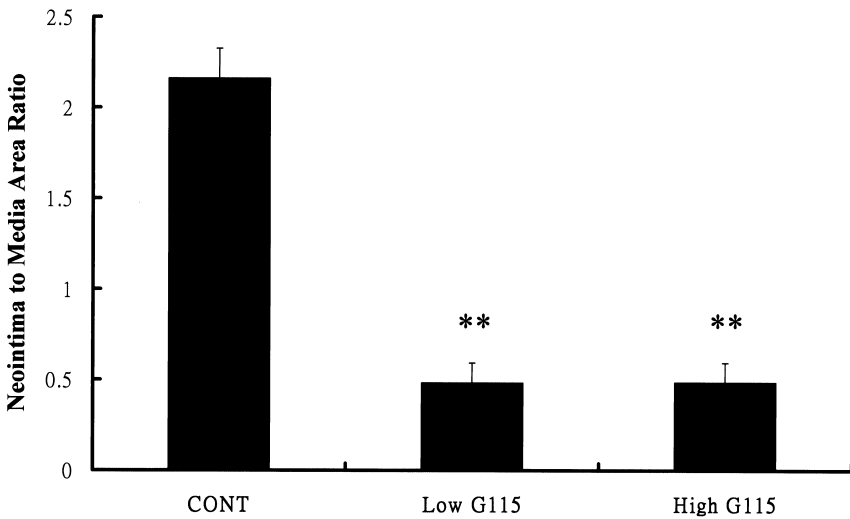


Fig. 5. The neointima to media area ratio in balloon injured rat carotid arteries. The control group shows a significant higher area ratio as compared to the one treated with low concentration (Low G115, 2mg/kg) and high concentration (High G115, 200mg/kg) of G115. Data are mean \pm SEM of 6 separate experiments. **P<0.01 as compared to the control group.

cology [24], Pezzuto proposed using plants or plant-derived preparations to prevent or inhibit carcinogenesis, suggesting a continuing growth of public enthusiasm for herbal medicine. However, its lack of specificity has made the virtues of ginseng skeptical to Western scientific and medical society. A standardized preparation of ginseng is therefore becoming necessary.

Smooth muscle cell proliferation, vascular remodeling, vascular contraction, and vascular oxidant stress are all believed to play key roles in the balloon injury-induced restenosis [25, 26, 27]. These processes can be grouped into two stages: a) a more acute loss of vasodilation capabilities, acute thrombosis mediated by loss of endothelial cells, augmented platelet and leucocyte activation and adhesion [1–3]; b) the late intimal formation and arterial remodeling [6]. Reagents or molecular interventions to suppress these factors may be beneficial in preventing such pathological condition. Ginsenosides, the active compounds of *Panax Ginseng*, have been reported to be effective in vascular relaxation by means of NO release from the endothelial cells [28]. G115, the standardized ginseng preparation, was also reported to have similar effects. Hearts from G115-treated rats perfused by the Langendorff technique showed significantly lower coronary vasoconstriction in response to angiotensin II than control hearts [29]. These results are in agreement with our *ex vivo* findings that G115 suppressed norepinephrine-induced vasoconstriction on rat thoracic aorta. Such vascular relaxation effects of G115 were found to be endothelium- and NO-dependent as endothelial denudation and L-NAME pretreatment prevented such relaxation as shown in figure 3. The vascular relaxation effects of G115 can be indicated in preventing balloon injury-induced stenosis.

In addition to vasorelaxation, ginsenosides was also reported to be an antioxidant. Cu, Zn-superoxide dismutase (Cu,Zn-SOD), a key enzyme involved in the metabolism of oxygen free radicals, was reported to be affected by ginsenosides [30]. With promoter analysis, it was found that specific binding of the AP2 transcription factor of Cu,Zn-SOD was significantly increased by ginsenosides. Since reactive oxygen species are thought to be the major mediators of signal transduction in vascular remodeling [20], a further experiment needs to be carefully designed to explore the effects of G115 on redox signaling pathway for restenosis.

Regarding the inhibitory effects of G115 on BrdU incorporation of smooth muscle cells, dead cells can be one of the important contributors for such reduction. However, apoptosis was not observed in the present study by G115 using either immunohistochemical or flow-cytometric analysis (data not shown). Equal amount of viable cells (10^6 cells) was used to determine the effects of G115 on BrdU incorporation. Oral administration of G115 in preventing balloon injury-induced stenosis in rats were in agreement with the *in vitro* inhibitory effects of G115 on BrdU incorporation. However, the gastric low pH environment may affect the activity of ginsenosides [17]. It was reported that ginsenosides Rg1 and Rb1 incubated at 37°C at pH1.2 for 1 hour followed by 5 hours incubation at pH 6.75 were much less effective to preserve ACh dilation following free-radical injury [17]. Nevertheless, the digested or undigested G115 dilated precontracted lung with perfusion and preserved Ach-induced dilation [17]. These results suggested that standardized extracts of ginseng G115 might contain certain components to preserve its pharmacological effects after oral administration than individual ginsenosides. This finding would support the oral ingestion of G115 prior to balloon injury as a preventive remedy for restenosis. However, the *in vitro* dose-dependent effect of G115 on cell proliferation was not found in the *in vivo* study. The initial strategy in the *in vivo* study was to use two doses of G115 for carrying out the experiments with a low dose (2 mg/kg)

100 times less than high dose (200 mg/kg). Surprisingly, there was a drastic reduction (77.3%) in intimal lesion even at low dose of G115. Because the *in vivo* study was performed by oral dosage, it would be appropriate to redesign a new experiment to investigate the effect of anti-neointimal formation using I.V. injection of G115. This experiment is now in progress to ultimately address this issue.

The *in vivo* assay of G115 on balloon injury-induced neointimal formation in the present study revealed an effective remedy in preventing vascular restenosis in animal models. Pre-treatment of G115 may provide a preventive therapy on vascular smooth muscle cell proliferation caused by balloon injury. Oral administration made this pharmacological therapy more convenient for those patients who are indicated for PTCA. To the best of our knowledge, the present study is the first report showing the preventive effects of ginseng extracts on balloon injury-induced stenosis. Although the precise doses of G115 to reach its optimal effects remained to be determined for clinical uses, this study provides a potential pharmacological therapy in preventing PTCA-induced restenosis.

Acknowledgments

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