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The dependence of nitric oxide synthase inhibition caused by cigarette smoking extracton the cellular aging of bovine aortic endothelial cells

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Objectives Cigarette smoking had been recorded as the main cause of impaired endothelium-dependent vasodilation in smokers by reducing nitric oxide (NO), a production of endothelial nitric oxide synthase (eNOS). However, the mechanism of NO impairment via eNOS activity is unclear until now. In this study, cell passage is suggested to be a relevant factor to eNOS expression under cigarette smoking stress.

Methods Bovine aortic endothelial cells (BAECs) were chosen as the research subject with passages ranking from 6 to 9 (6P to 9P). After exposure of cigarette smoking extract (CSE) solution, MTT assay and Western blot method were performed to check the cell viability as well as eNOS protein concentration. In these experiments, four concentrations of CSE at 0.5, 1, 2, and 4% were selected for treatment.

Results Our results showed that cells almost died at 4% of CSE. Besides, eNOS protein mass had a linear decrease under the increase of CSE concentration. In addition, the effect of CSE on eNOS expression was dissimilar between different passages.

Conclusions This study indicated that CSE had effect on both cell viability and eNOS expression. Besides, a reduction in protein mass was matched with the decrease of cell viability due to CSE tress. Last but not least, the response of eNOS protein to different concentration of CSE at different passages was disparate, making the hypothesis about cell passage related inhibition of eNOS caused by CSE solution.

Keywords Bovine aortic endothelial cells, Cellular aging, Cigarette smoking extract, Endothelial nitric oxide synthase

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Introduction

Cigarette smoking was long time known as a risk factor in atherosclerosis, loss pulmonary function and related cardiovascular disease due to the contain of many toxic chemicals [1]. An evidence about cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation was recorded in healthy young adults [2]. Endothelial-derived relaxing factors were found and researched from soon for application in vascular related diseases, and nitric oxide (NO) is among of them [3]. NO is an important molecule in vasculature, it was first time identified as an endothelial-derived relaxing factor in 1988 [4] and studied its role in endothelial function later [5]. NO acts as an endogenous nitrovasodilator, stimulating soluble guanvlate cyclase to increase cyclic guanosine monophosphate levels in vascular smooth muscle and platelets, with consequent relaxant and anti-aggregatory effects [6]. The vascular endothelial cells synthesize NO from L-arginine to via the catalytic reaction of endothelial nitric oxide synthase (eNOS). Some studies figured out that cigarette smoking extract (CSE) decreased exhaled NO, suggesting that it may inhibit the enzyme NO synthase [7]. This question was answered

later in another research to confirm the inhibition of CSE on eNOS activity is irreversible [8].

However, the mechanism and the effective factors of this process were not clearly understood until now. Cell passage is also an important factor in cell study. Some authors just tested the eNOS expression in endothelial cells at identical passage and no one indicated the connection between cell passage and eNOS expression under CSE stress. So we did this study to answer the question above. Our results showed that CSE had dose-dependent effect on eNOS inhibition and emphasized that this inactivation of eNOS related to cell passage, which was not mentioned before.

Materials and Methods

Cell Culture

Endothelial cells were collected from the cardiac aortic of the 30 months-old cows and maintained in minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 5% newborn calf serum, L-glutamine and 1% penicillin–streptomycin solution in humidified atmosphere incubator containing 5% CO_2 at 37°C. The 6th- to 9th-passage cells using for all experiments were grown in 100 mm dishes with monolayer and changed medium every 2 days.

Cigarette Smoking Extract Preparation

This, commercial cigarettes purchased from KT&G Corporation was used to prepare CSE solution. For generating 100% CSE solution, 3 cigarettes were smoked continuously using a vacuum to adsorb all smoke into an erlen contain 30 mL of Dulbecco's phosphate buffered saline that was pre-warmed before. The whole solution then was filtered using 2 mm Cambridge filter to generate the gas-phase extract solution [8].

MTT Assay

MTT assays were carried out on different cell passaged to assess the cell viability under the stress due to CSE treatment at various concentrations (0.5, 1, 2, and 4%). One hundred percent CSE solution prepared in previous step was diluted in treating samples to get the final concentrations. Cells were grown in 96 wells plate with the density of 3×10^3 cell/well and exposed to CSE solution within 24 hours. Three replicates were performed for each concentration. The absorbance of colored solution was quantified by measuring at 560 nm wavelength using Elisa reader (Thermo Electron Co., Shanghai, China) [9].

Total Protein Isolation

To prepare protein samples for western blot analysis, cells were starved within 12 hours before a 24 hours CSE solution exposure. After 1 day of treatment, cells from each 100 cm dish were lysed in a mixture of lysis buffer (20 mM tris-Cl pH 7.4, 1 mM EDTA pH 8.8, 150 mM NaCl, 1 mM EGTA pH 8.5, 1% v/v Triton X-100), protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and phenylmethanesulfonyl fluoride (Roche) with ratio 100:1:1 using scrappers. Lysates were then agitated by vortex mixer for 10 minutes (at 10 seconds period) and incubated in ice during 30 minutes prior to being centrifuged at 13,000 rpm, 4°C for 10 minutes to yield a supernatant [10].

Western Blot

We applied western blot method to check the expression of eNOS protein under the treatment of CSE solution. Protein concentrations were measured by Bradford assay using Elisa reader machine (Thermo Electron). An amount of 20 μg protein was loaded into each well in SDS-PAGE followed by the step of electronic transferring to nitrocellulose membrane. The protein-membrane system was then blocked in 5% skim milk for 1 hour and incubated over night at 4°C with the appropriate primary antibody as followed: eNOS and β-actin (as control) with ratio of dilution is 1:500. Subsequently, the membranes were washed with wash buffer and incubated with respective secondary antibody for 1 hour at room temperature. Antibody complexes were visualized by enhanced chemiluminescence using ECL kit (Elpis Biotech, Daejeon, Korea) [11].

Data Analysis

All data was obtained from three independent samples carried out simultaneously for error analysis. The results are reported along with the standard deviation. In addition, the correlation between the cell viability and several experimental conditions is also reported. The statistical significance was determined using a Student's *t*-test for two points. The *p*-values < 0.05 were considered significant.

Results

Toxicity of Cigarette Smoking Extract Solution Effect Cell Viability

The bovine aortic endothelial cells (BAECs) almost were not attached into the plastic dishes when treated with higher than

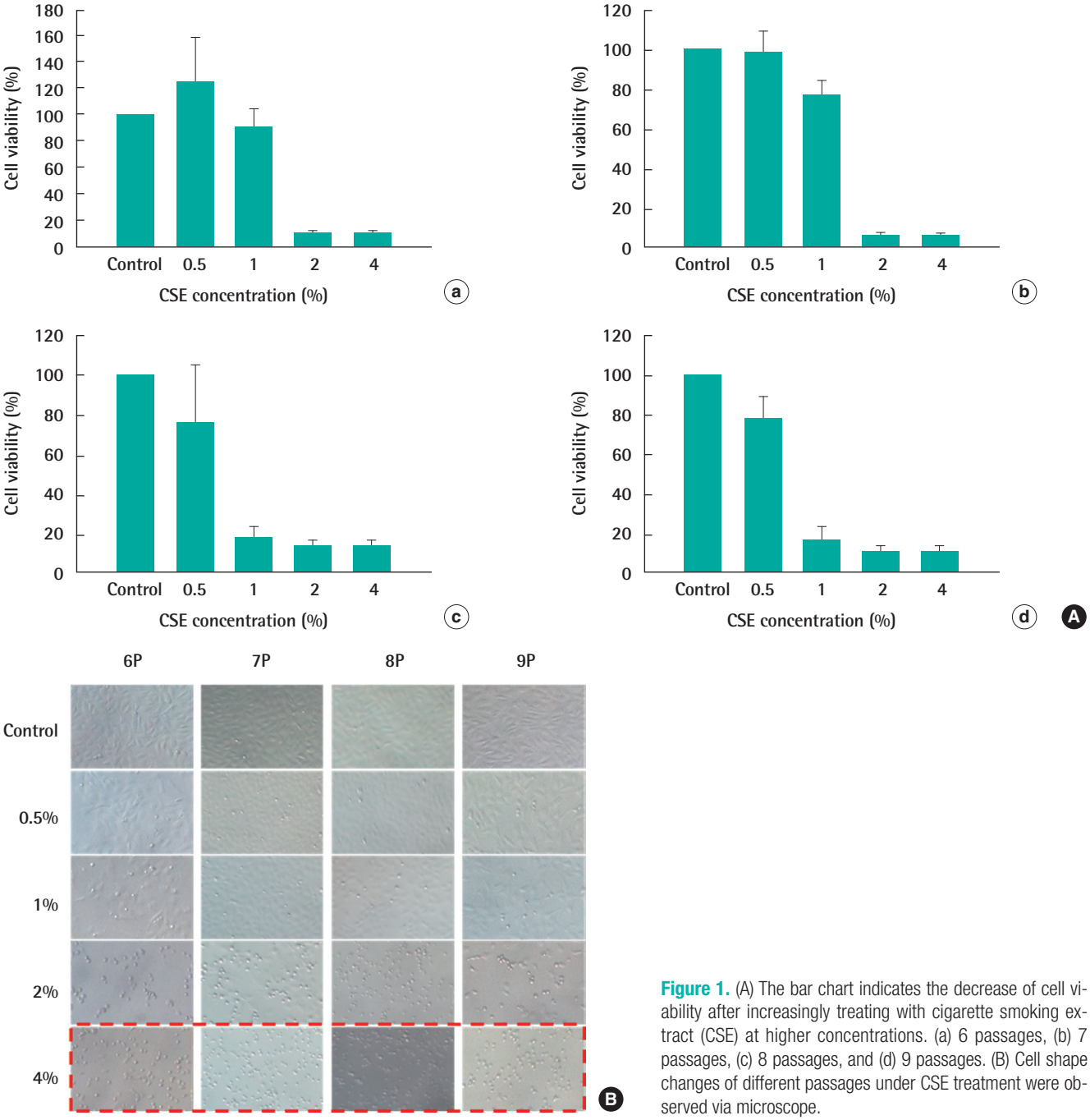

Figure 1. (A) The bar chart indicates the decrease of cell viability after increasingly treating with cigarette smoking extract (CSE) at higher concentrations. (a) 6 passages, (b) 7 passages, (c) 8 passages, and (d) 9 passages. (B) Cell shape changes of different passages under CSE treatment were observed via microscope.

2% of CSE solution and absolutely floated in medium at 4% CSE. As shown in Figure 1, the graphs illustrate the correlation between the cell viability and the CSE concentration. The higher concentration of CSE we treat, the more cells die. This means the BAECs showed its tolerance to CSE solution decreasingly along with the increase of CSE concentration in culture medium comparing to the control cells. Besides, the protein quantification result also matched with MTT result of each cell passage (Figure 2). Total protein concentration was appropriate zero at 4% CSE.

The Effect of Cigarette Smoking Extract on the eNOS Expression

The effect of CSE solution on the expression of eNOS synthase enzyme was confirmed by western blot results (Figure 3). At the 6th and 7th passage, eNOS showed the up-regulation when increasingly treated with higher concentration of CSE. Meanwhile cells from higher passage (8th and 9th passage) went on an inverse result that the expression of eNOS protein reduced along with the CSE concentration go-up-ward (Figure 3). Additionally,

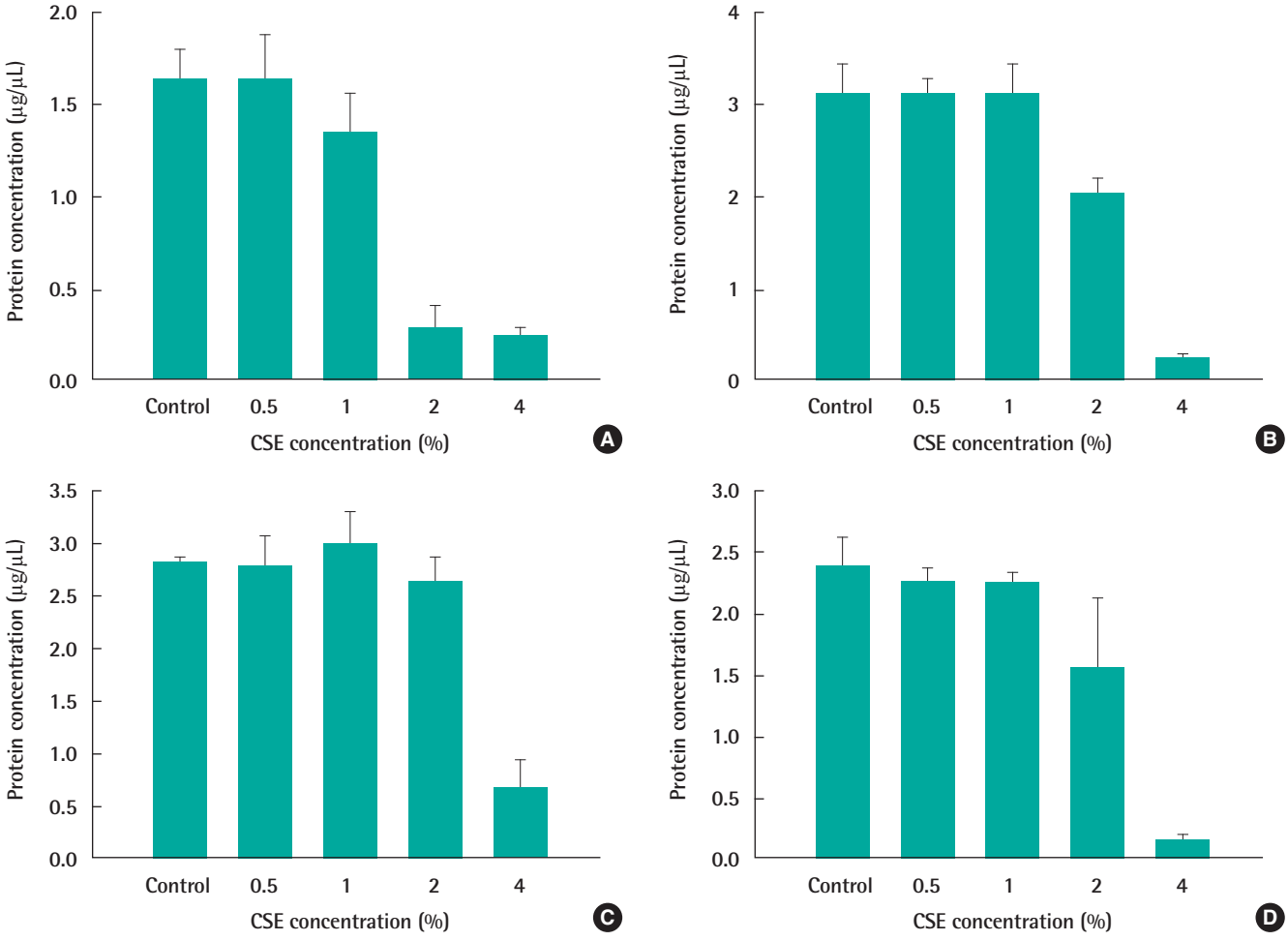

Figure 2. The linear induction of protein mass in all tested cell passages resulted by the growth of cigarette smoking extract (CSE) percentage in treated medium. (A) 6 passages, (B) 7 passages, (C) 8 passages, and (D) 9 passages.

most of 4% CSE treated samples did not have the β-actin bands because of very low total protein concentration due to lost (death) cells.

Discussion

This study demonstrates that exposed BAECs from 6th to 9th passage with CSE solution in dose-dependent has a linear change in cell viability, protein content, and eNOS expression. When treated with CSE in increasing ward of concentration, in all passage stages, cell adhesion went down leading to the loss of protein content with large amount (2% and 4% of CSE), indicating that the changes in eNOS protein mass is responsible. May be the CSE components absorbed into cells and induced apoptosis of BAEC as the mechanism that once described in previous study [12]. However, necrosis should be suggested for the reason. A study in 2003 carried out the opposite results that cigarette smoke induces necrosis in alveolar type II cells, endothelial cells, and Jurkat cells. Moreover, cigarette smoke condensate inhibited caspase (an essential protease for apoptosis) activation and apoptosis in A549 and Jurkat cells. This indicates that necrosis but not apoptosis in response to cigarette smoking is significant [13].

The important thing to discuss here is how cells responded to CSE in different cell passages. They did not show the same feedback to CSE exposure as previous suggestion that eNOS content would decrease. Reversely, we can separate into two dissimilar ways of eNOS response: up-regulation in early passages (6th and 7th passage) and down-regulation in later passages (8th and 9th passage). This can be explained by cell passage-dependent regulation system that never be recorded yet in previous studies about CSE related disease in endothelial cells. We suggest that at lower passages, eNOS protein has stronger response to CSE tress for increasing intracellular NO level. In details, at passage 6 and 7, eNOS expressed more strongly before was down-regulated or inactivated at high level of CSE while there was just one downward of manipulation in eNOS expression at passage 8 and 9 (Figure 3). In another words, eNOS synthase is more sen-

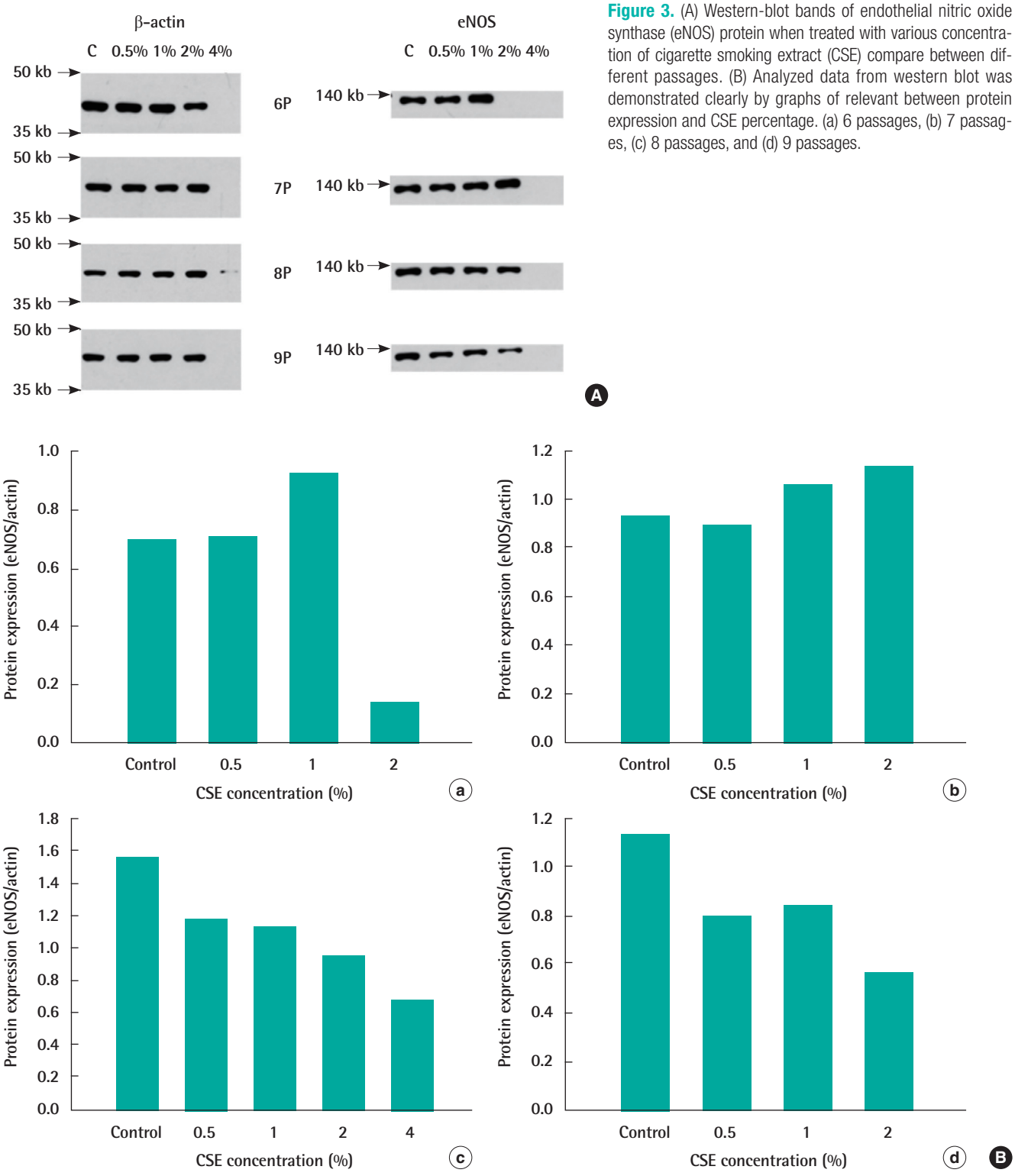

Figure 3. (A) Western-blot bands of endothelial nitric oxide synthase (eNOS) protein when treated with various concentration of cigarette smoking extract (CSE) compare between different passages. (B) Analyzed data from western blot was demonstrated clearly by graphs of relevant between protein expression and CSE percentage. (a) 6 passages, (b) 7 passages, (c) 8 passages, and (d) 9 passages.

sitive with CSE at lower passages more than at later stages. The inhibition of eNOS expression in our result is also matched with other authors which can be reasoned by the negative - feedback regulation of eNOS expression by exogenous NO due to CSE solution [14]. Continuous low dose inhaled NO has good effect on vascular disease [15], but if smoked cigarette too much, patients would absorb extra NO into endothelial cells because of NO included in CSE constituents [16].

In summary, our work investigated the effect of CSE solution at various level of concentration on bovine aortic endothelial cells. The results indicate that CSE have dose-dependent influence on cell viability, protein mass as well as eNOS expression,

and they themselves matched each other. Besides, the inhibition of eNOS protein had concern with different cell passages. In earlier stages (6th and 7th passages), the inactivation of eNOS just appeared at high concentration of CSE whereas it started from low level of CSE in later stages (8th and 9th passage). This was first time described here to support the theory of research about the effect of CSE on endothelial derived vascular disease. Further experiments need to be done to confirm the NO concentration after exposing to CSE, implementing to the results found in this study.

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Conflict of Interest

The authors have no conflicts of interest with material presented in this paper.

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