

Original Article

The influence of nitroglycerin on the proliferation of endothelial progenitor cells from peripheral blood of patients with coronary artery disease

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Endothelial progenitor cells (EPCs) are associated with vascular repairing and progression of atherosclerotic lesion. It may lead to coronary artery disease (CAD) if circulating EPCs lose their function. Continuous nitroglycerin (NTG) therapy causes increased vascular oxidative stress and endothelial dysfunction. The aim of this study was to investigate the effects of NTG on the proliferation of human peripheral blood-derived EPCs. EPC cultures, collected from 60 CAD patients and cultured for 7–12 days, were treated with different concentrations of NTG (0.0, 0.3, 1.0, 2.0, 7.5, 15.0, and 20.0 mg/l) for 72 h, respectively. The cell counts and proliferative activities of EPC; the levels of vascular endothelial growth factor-A (VEGF-A), nitric oxide (NO) and peroxynitrite (ONOO⁻) in culture medium; and the level of reactive oxygen species (ROS) in adherent cells were measured. Compared with control (0.0 mg/l NTG), the cell number and proliferative activities of EPCs were increased when treated with 1.0 mg/l NTG and reached maximum level when NTG concentration was 7.5 mg/l. However, there was a significant reduction when treated with higher doses of NTG (≥ 15.0 mg/l). Meanwhile, VEGF-A expression reached its maximal expression with 7.5 mg/l NTG, but gradually declined by incubation with higher doses of NTG. There was a linear relationship between NO level and NTG concentration, but no changes of ONOO⁻ and ROS levels were found when EPCs were incubated with 0.3–7.5 mg/l NTG. However, ONOO⁻ and ROS levels were significantly increased when incubated with 15 and 20 mg/l NTG. Our data demonstrated that moderate dose of NTG may stimulate the proliferative activities of EPCs isolated from CAD patients.

Keywords nitroglycerin; endothelial progenitor cell; proliferation; nitric oxide

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Introduction

It is well known that vascular endothelium plays an important role in maintaining cardiovascular homeostasis. More evidence showed that endothelial dysfunction is the main factor for the initiation and progression of atherosclerosis and coronary artery disease (CAD) [1–3]. Endothelial progenitor cells (EPCs) are peripheral blood (PB)- or bone marrow (BM)-derived cells that will differentiate into functional mature endothelial cells under specific conditions [4,5]. EPCs may be essential in promoting the repair of damaged endothelium and inhibit the progression of atherosclerotic lesion [6–8]. The proliferation of circulating EPCs is correlated with coronary endothelial function. Low cell counts of circulating EPCs were found in patients who have already developed coronary heart disease [9]. Moreover, inadequate coronary collateral development has been shown in patients with EPC dysfunction [10].

Nitric oxide (NO) has been suggested to be a pathophysiological regulator for cell proliferation, cell cycle arrest, and apoptosis [11]. Several pieces of evidence suggested that NO plays an essential role in keeping the normal migration and reparative function of EPCs [12–14]. Ozüyaman *et al.* [15] reported that under NO-deficient conditions, granulocyte-colony stimulating factor (G-CSF) failed to increase cell counts of EPCs that were derived from BM. Moreover, higher NO bioavailability and endothelial NO synthase expression were observed in PB-derived human EPCs (hEPCs) cultured for 14 days than in those cultured for 3 days [16]. Thus, NO may have important impact on EPC proliferation.

In addition, NO was endogenously synthesized from the oxidation of L-arginine to L-citrulline by the action of endothelial NO synthase (eNOS) [17]. With aging and vascular disease, endothelium gradually decreased the NO releasing capacity [18]. Tissue hypoxia was found to inhibit the

endogenous NO synthesis [19]. The impaired endothelial regeneration processes were observed in patients with ischemic heart disease who were characterized by a reduced systemic NO bioactivity [20]. It is likely that other sources of NO become more important. Nitroglycerin (NTG) is a well-known antianginal agent and often used under conditions of cardiovascular ischemia. As a NO donor drug, NTG has been suggested to be metabolized to NO and supplement exogenous NO [21]. However, data concerning the administration of exogenous NO generated by NTG in EPC proliferation in CAD are not available.

In this study, we investigated the effects of various concentrations of NTG on the proliferation of EPCs isolated from CAD patients.

Materials and Methods

Samples

In the present study, informed consent was obtained from all patients based on a protocol approved by the Ethics Committee of Qilu Hospital of Shandong University (Number: KYLL-2013-115). We prospectively studied 60 patients with a suspicion of CAD who were admitted for elective coronary angiography to the Department of Cardiology, Qilu Hospital of Shandong University.

Isolation and culture of putative EPCs

Human PB (50 ml each) was collected from patients for the isolation of mononuclear cells (MNCs). MNCs were isolated by density gradient centrifugation using Biocoll (Biochrom, Berlin, Germany) for 20 min at 500 *g* and washed three times in phosphate buffer solution (PBS; Biochrom). Cells were seeded at a density of $(2-5) \times 10^6$ /ml in medium 199 (HyClone, Logan, USA), supplemented with 20% fetal bovine serum (FBS; Sigma, St Louis, USA). Then cells were coated with human fibronectin (ChemiCon, Temecula, USA). Human MNC cultures were maintained in a humidified incubator under an atmosphere of 5% CO₂ and 95% air at a constant temperature of 37°C.

After cells were cultured for 7 days, immunofluorescence staining was carried out to further identify EPCs. Cells were incubated with 2.4 mg/l 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate labeled acetylated low-density lipoprotein (Dil-ac-LDL) (Invitrogen, Carlsbad, USA) at 37°C for 1 h. Then cells were washed twice with PBS-bovine serum albumin (BSA) and fixed with 4% paraformaldehyde for 15 min. After being washed three times with PBS-BSA, cells were incubated with 10 mg/l fluorescein isothiocyanate (FITC)-conjugated lectin from *Ulex europaeus* (FITC-UEA-I) solution (Sigma) for 1 h. Only double-positive (Dil-ac-LDL and UEA-I) EPCs were mounted and photographed with a fluorescent microscope (Olympus Deutschland GmbH, Hamburg, Germany).

EPC proliferation assay

The effect of NTG on EPC proliferation was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After being incubated for 7 days, EPCs were treated with 0.25% trypsin and then cultured in serum-free medium in 96-well culture plates (200 μ l/well). Different concentrations of NTG (0, 0.3, 1.0, 2.0, 7.5, 15.0, and 20.0 mg/l) ($n = 6$ wells) were added into the medium, respectively. At 72 h, 20 μ l of MTT solution was added to each well and incubated for another 4 h. Then, the supernatant was removed by suction, and EPCs were incubated with 100 μ l dimethyl sulfoxide for 10 min. The optical density (OD) value was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, Waltham, USA).

After being cultured for 12 days, the effects of NTG on the cell counts of EPCs and EPC colony were determined by two independent blinded investigators under high power field using a microscope (CX31; Olympus, Tokyo, Japan). Results were expressed as cells or colonies per mm² [22]. Spindle-shaped cells and colonies were counted. EPC colony was manually counted in eight random microscopic fields ($\times 400$) in 24-well culture plates.

Measurement of NO and ONOO⁻ levels in culture medium

Cultured EPCs were incubated with NTG at various concentrations of 0.0 (as control), 0.3, 1.0, 2.0, 7.5, 15.0 and 20.0 mg/l for 72 h, respectively. Then, the supernatants were harvested and used for nitrite determination using an NO kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, 50 μ l of the supernatant was mixed with 50 μ l Griess Reagent I (0.2% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) and 50 μ l Griess Reagent II (2% sulfanilamide in 5% phosphoric acid). NO levels in culture media were calculated from the absorbance at 540 nm. ONOO⁻ was measured in the supernatants using an ONOO⁻ detection kit (TransGen Biotech Co. Ltd., Beijing, China) according to manufacturer's instructions.

Measurement of ROS in EPCs

Reactive oxygen species (ROS) levels generated by the adherent EPCs were measured when NTG was prepared at concentrations of 0.0, 7.5, and 20.0 mg/l. ROS levels were detected using the redox-sensitive dye chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA) (Life Technologies GmbH, Darmstadt Germany). Cultures were incubated in the appropriate experimental medium containing CM-H2DCFDA (10 μ M) for 15 min at 35°C. Excitation and emission peaks were found at 502 and 530 nm, respectively.

Measurement of VEGF-A expression in culture medium

As described above, EPCs were incubated with NTG at various concentrations of 0.0 (as control), 0.3, 1.0, 2.0, 7.5, 15.0, and

20.0 mg/l. Samples were centrifuged for 20 min at 1000 g to separate cell pellets, and the supernatants were collected after 72 h of incubation. Vascular endothelial growth factor-A (VEGF-A) expression in the supernatants was measured by a commercial ELISA kit (R&D System, Minneapolis, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm.

Statistical analysis

All data are expressed as the mean \pm standard deviation. After testing for normal distribution of variables, the one-way analysis of variance test was used for repeated measures. Statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, USA) software. A *P* value of <0.05 was considered statistically significant.

Results

Characterization of EPCs

After isolation, MNCs were plated on fibronectin-coated culture dishes and cultured for 14 days. At day 7, the plates were scanned for the apparent presence of EPC colonies in the inverted microscope. EPCs were elongated and had a spindle shape (**Fig. 1A**). After 14 days of culture, cells

mainly formed large EPC colonies [23], which were composed of spindle-appearing cells at the periphery (**Fig. 1B**).

Also at day 7, results from the fluorescent microscope showed that almost all of the MNC-derived EPC colonies were double-positive for FITC-UEA-I and DiI-Ac-LDL. Cultured EPCs bound to the specific FITC-UEA-I showed green fluorescent aggregates (**Fig. 1C**), and cultured EPCs swallowed DiI-ac-LDL showed red fluorescent aggregates (**Fig. 1D**).

Evaluation of EPC proliferation

The effects of NTG on the proliferative capacity of EPCs were investigated by MTT assay. **Figure 2** showed the dose-response curve of the OD values of EPCs incubated with NTG for 72 h. There is a significant increase of EPC proliferative activity that starts from 1.0 mg/l NTG ($P < 0.05$) and reaches a maximum level at 7.5 mg/l NTG ($P < 0.01$) when compared with the control (0.0 mg/l NTG). However, when treated with a higher dose of NTG (15.0 mg/l), there was a reduction in EPC proliferative capacity compared with control ($P > 0.05$). A further significant decrease was observed when the dose of NTG reached 20.0 mg/l ($P < 0.01$).

To validate this result, manual counting of EPCs was also carried out (**Fig. 3**). After the intervention of different concentrations of NTG for 72 h, the number of EPCs and EPC

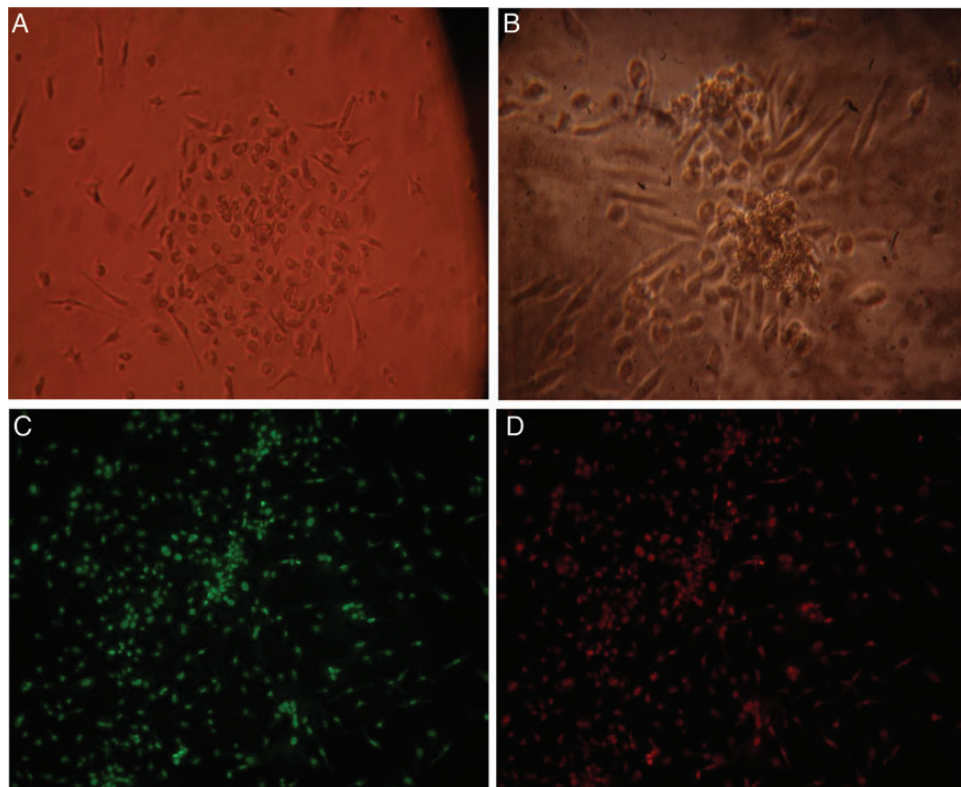


Figure 1. Characterization of EPCs (A,B) The morphology of EPCs at different culture periods. (A) After 7 days of culture, cells formed apparent colonies (100 \times). (B) After 14 days of culture, EPCs were grown to confluence and show spindle-appearing cells at the periphery (400 \times). (C,D) EPC characterization by immunofluorescence staining. (C) Cultured EPCs bound to the specific FITC-UEA-I showed green fluorescent aggregates. (D) Cultured EPCs swallowed DiI-ac-LDL showed red fluorescent aggregates.

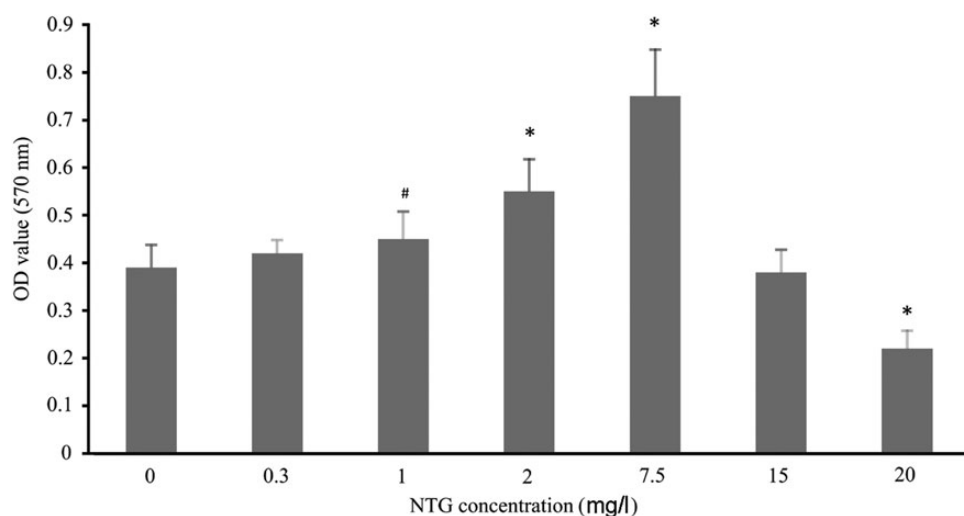


Figure 2. Effects of NTG on EPC proliferation measured by MTT assay An increase in EPC proliferative activity at 1.0 mg/l NTG became significant and reached maximum level at 7.5 mg/l NTG when compared with control (0.0 mg/l NTG). When treated with higher dose of NTG (15.0 mg/l), there was a reduction in EPC proliferative capacity compared with control. A further significant decrease was observed when the dose of NTG reached 20.0 mg/l. # $P < 0.05$ vs. control, * $P < 0.01$ vs. control.

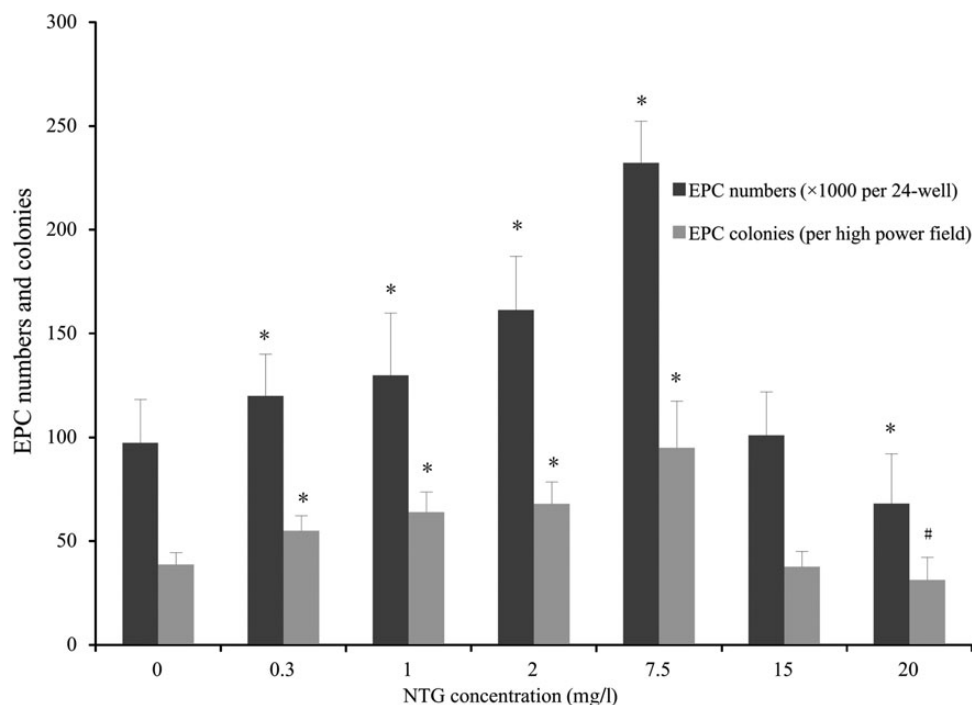


Figure 3. Effects of NTG on EPC number and EPC colonies determined by a microscope Treated with different concentrations of NTG for 72 h, the number of EPCs and EPC colonies was significantly increased, and the highest effect was achieved at a dose of 7.5 mg/l. However, when treated with higher dose of NTG (≥ 15.0 mg/l), there was no significant increase in the number of EPCs and EPC colonies when compared with control. # $P < 0.05$ vs. control, * $P < 0.01$ vs. control.

colonies was significantly increased, and the highest effect was achieved at a dose of 7.5 mg/l (all $P < 0.01$). However, when treated with higher dose of NTG (15.0 mg/l), there was no significant increase in the number of EPCs and EPC colonies compared with control (all $P > 0.05$). Further decreases were observed when the dose of NTG reached the maximum dose (20.0 mg/l) ($P < 0.01$ for number of EPCs; $P < 0.05$ for EPC colonies).

NO and ONOO⁻ levels in culture medium

The level of NO was increased when NTG concentration was increased in the culture medium (all $P < 0.01$). However, compared with control (0.0 mg/l NTG), no statistically significant trend in ONOO⁻ level was observed when EPCs were incubated with 0.3, 1.0, 2.0, and 7.5 mg/l of NTG (all $P > 0.05$). The results showed that higher concentration of NTG (15 mg/l) caused greater increase of

Table 1. Effects of different concentrations of NTG on NO, ONOO⁻, ROS, and VEGF-A

NTG concentration (mg/l)	NO (μ M)	ONOO ⁻ (ng/l)	ROS	VEGF-A (ng/l)
0.0	75.96 \pm 10.74	6.85 \pm 1.36	30.89 \pm 2.23	70.97 \pm 14.77
0.3	208.09 \pm 37.45**	7.02 \pm 0.66	–	91.72 \pm 13.38**
1.0	268.11 \pm 59.25**	7.34 \pm 0.90	–	98.63 \pm 8.71**
2.0	361.80 \pm 74.73**	7.43 \pm 0.90	–	100.29 \pm 9.30**
7.5	720.83 \pm 91.04**	7.46 \pm 0.88	39.03 \pm 5.44	115.23 \pm 12.00**
15.0	1209.69 \pm 199.43**	8.22 \pm 0.70**	–	70.44 \pm 13.65
20.0	1666.24 \pm 277.04**	8.97 \pm 0.79**	60.18 \pm 9.49**	60.18 \pm 9.49**

Values are expressed as the mean \pm SD; * P < 0.05 vs. control, ** P < 0.01 vs. control.

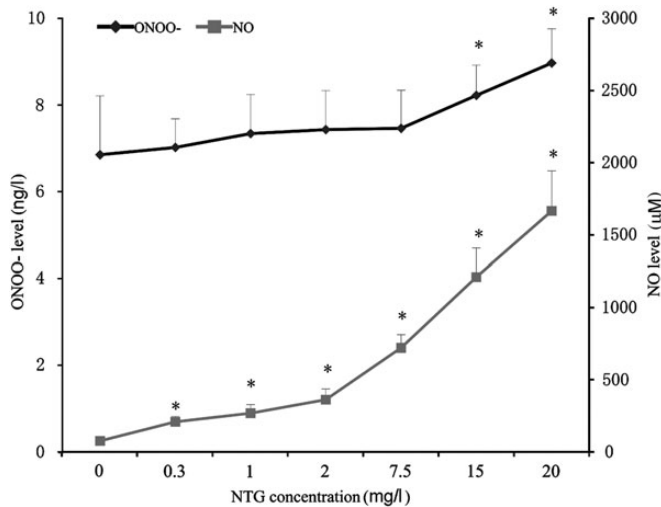


Figure 4. Effects of NTG on NO and ONOO⁻ levels in culture medium Incubation with NTG dose-dependently increased level of NO in the culture supernatants. Meanwhile, significant increases were observed in ONOO⁻ level at the high doses tested, 15 and 20 mg/l. * P < 0.01 vs. control.

detectable ONOO⁻ (P < 0.01). A further significant increase of ONOO⁻ level was observed at the highest doses tested, 20 mg/l (P < 0.01) (Table 1 and Fig. 4).

ROS level in adherent EPCs

Compared with control (0.0 mg/l NTG), treatment with 20 mg/l of NTG increased ROS synthesis in adherent cells (P < 0.01), whereas treatment with 7.5 mg/l of NTG had no effect (P > 0.05) (Table 1 and Fig. 5).

VEGF-A expression in culture medium

VEGF-A protein expression was confirmed by enzyme-linked immunosorbent assay (ELISA). As shown in Table 1 and Fig. 6, the VEGF-A level in supernatants harvested from cultured EPC was increased after incubation with NTG. Compared with control, maximal VEGF-A level was detected at 7.5 mg/l of NTG (P < 0.01), followed by a gradual decline after incubation with 15.0 mg/l of NTG (P > 0.05). A

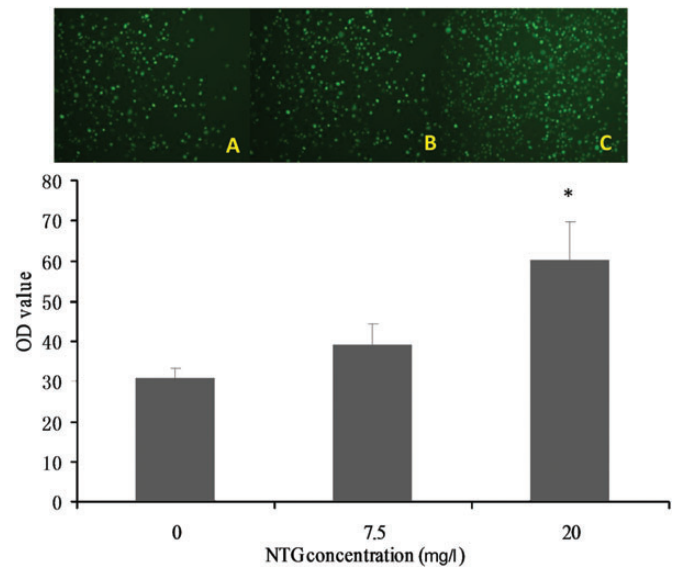


Figure 5. Effects of NTG on ROS in adherent EPCs The above images obtained from the fluorescence microscope showed green fluorescence, indicating the generation of ROS in adherent EPCs (A, B and C respectively represent 0, 7.5 and 20 mg/l NTG treatment). The below histograms showed that 20 mg/l of NTG treatment significantly increased ROS production when compared with control. * P < 0.01 vs. control.

significant down-regulation of the VEGF-A level (P < 0.05) was observed when incubated with 20.0 mg/l of NTG.

Discussion

Our results suggested that (i) the proliferation of EPCs was stimulated by moderate concentrations of NTG (\leq 7.5 mg/l) and inhibited by higher concentrations of NTG (\geq 15 mg/l); (ii) the NO concentration in the culture medium increased with increasing NTG concentration; (iii) moderate concentrations of NTG (\leq 7.5 mg/l) had no effect on ONOO⁻ level in supernatants, but higher concentrations of NTG (\geq 15 mg/l) increased ONOO⁻ level; (iv) ROS production was increased when exposed to the highest concentrations of NTG (20 mg/l) in adherent cells; and (v) the expression of VEGF-A was

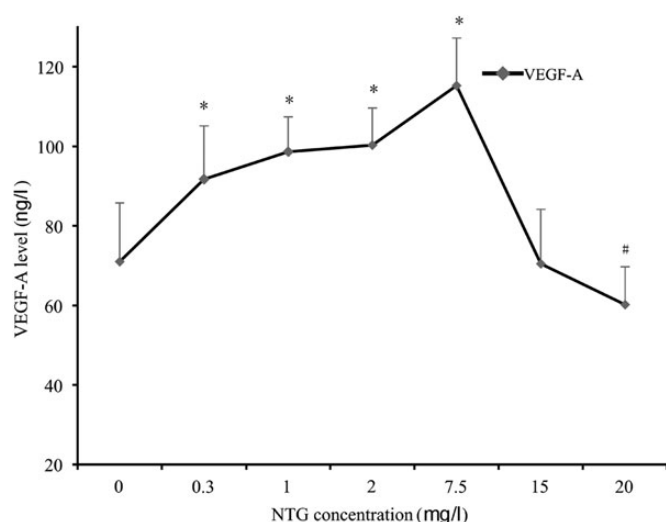


Figure 6. Effects of NTG on VEGF-A level in culture medium Maximal VEGF-A level was detected at 7.5 mg/l of NTG, followed by a gradual decline after incubation with higher dose of NTG (≥ 15.0 mg/l) when compared with control. # $P < 0.05$ vs. control, * $P < 0.01$ vs. control.

increased when exposed to moderate concentrations of NTG (≤ 7.5 mg/l), but will be inhibited with higher concentrations of NTG (≥ 15 mg/l). These novel findings suggested that the amount of NO released from moderate concentrations of NTG contributed to the proliferative capacity of EPCs. However, excessive ROS and ONOO⁻ levels were associated with the reduction of VEGF-A expression and the proliferation of EPCs with higher concentrations of NTG.

EPCs are considered as highly potent regenerating cells with high proliferation ability in response to tissue ischemia or hypoxia. EPCs were found to be essential in promoting the repair of damaged endothelium and to inhibit the progression of atherosclerotic lesion [6–8]. Some studies showing that EPCs were *in vitro* isolated from PB or BM have been published based on some interventional clinical trials in cardiovascular system [24]. When EPCs were intravenously injected to ischemic areas, they can differentiate into new blood vessels in the infarct-bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) [25]. There has been some success in using EPCs as therapeutic agents. However, EPCs has not been successfully used in the *in vivo* systemic transplantation because of some limitations of EPCs, such as poor biodistribution, low cell survival, distinct populations usage, and different cell delivery duration and routes [24,26,27]. It is recognized that continuous treatment with NTG was associated with endothelial dysfunction [28]. Thus, NTG therapy may affect EPCs. DiFabio *et al.* [29] reported that NTG exposure affected differently on apoptosis, phenotypic differentiation, or migration of EPCs *in vivo* and *in vitro*. In the present study, we demonstrated a critical role of different concentrations of NTG in the proliferation of EPCs that were isolated

from PB. We observed a significant increase of EPC proliferation with moderate doses of NTG, whereas higher concentrations of NTG therapy may be toxic. Although the concentrations of NTG used in the present study exceeded the upper limit of NTG administration dosage *in vivo* [30], our results suggest a potential role of NTG in cultured EPCs.

The underlying mechanisms of how NTG regulates EPC proliferation are not clear, but bioavailability of NO or ROS synthesis may be involved. NO is endogenously synthesized from the oxidation of L-arginine to L-citrulline by the action of eNOS [17]. The constitutive eNOS in the arterial endothelium continuously generates NO, which has been shown to regulate vascular relaxation and inhibit vascular smooth muscle cell proliferation, platelet adhesion and aggregation, and monocyte adhesion and migration [31]. Consequently, NO may protect arterial endothelium from atherosclerosis and thrombosis. However, excessive NO is toxic and may lead to many chronic diseases such as atherosclerosis, diabetics, post-ischemic perfusion injury, myocardial infarction, chronic inflammation, and cancer [32]. It has been suggested that continuous exposure to NTG within 24–48 h increases the vascular response to ROS, such as superoxide anion (O₂⁻) and ONOO⁻, and causes endothelial dysfunction [28,33]. The interaction between O₂⁻ and NO released from NTG could result in the increase of ONOO⁻ formation. ONOO⁻ causes eNOS uncoupling (thus further reduced NO and increased O₂⁻ generation) and leads to, ultimately, nitrate tolerance [28,34]. In the present study, after continuous exposure to NTG for 72 h, the EPC proliferative capacity was increased with the increase of NO released from NTG. Despite the observed NO released from NTG in a dose-dependent manner, there was a significant decrease in the number of EPCs at 15–20 mg/l of NTG. Our studies further showed that ONOO⁻ generation was increased in EPCs when treated with 15 mg/l of NTG, suggesting that increased ROS levels and overproduction of ONOO⁻ indeed resulted in the proliferation impairment of EPCs. These findings suggested that decreasing ROS production would restore the effectiveness of therapeutic tools that enhance proliferative activity of EPCs.

We also observed the elevated VEGF-A expression in the culture medium of EPCs, whereas VEGF-A level was decreased with higher concentrations of NTG. The change of VEGF-A expression was related to the proliferative activity of EPCs. Differentiation, mobilization, and recruitment of EPCs have been established to be regulated by several angiogenic factors, such as VEGF [4,35]. Furthermore, a previous report suggested that NO appears to be a downstream mediator of VEGF [36]. VEGF treatment increases the activation of eNOS and NO synthesis is required at the site of vessel formation by EPCs, which also promotes the proliferative capacity of human EPCs [37]. VEGF is expressed in many cell types, notably parenchymal cells

adjacent to capillaries and vascular cells adjacent to endothelia [38,39]. Rehman *et al.* [40] reported that cultured EPCs did not show any significant proliferation but did secrete the angiogenic growth factors, such as VEGF. Our findings that were different from theirs [40] demonstrated that an increase of VEGF-A protein accompanies with the increase of EPC proliferation. In our study, the adequate ROS production may be helpful rather than harmful to VEGF-A protein secreted from EPCs in the presence of moderate concentrations of NTG. NO and ONOO⁻ levels were increased when EPC was incubated with higher doses of NTG, which is accompanied with a decrease in VEGF-A level. ROS plays a critical role in facilitating the angiogenic signal of VEGF in vascular cells [41]. Abdelsaid *et al.* [42] reported that the oxidative stress inhibited the VEGF-mediated angiogenic response. Consistent with these results, our findings suggested that excessive ROS and ONOO⁻ production may affect VEGF-A protein secreted from cultured EPCs *in vitro*.

Some limitations of this study need to be mentioned. Firstly, this study focused on the NTG-induced proliferation of EPCs *in vitro* instead of *in vivo*. Secondly, we only assessed the effects of NTG on EPC proliferation; accordingly, and cannot answer the question whether there are differences in EPC function. Finally, NTG-induced impairments of endothelial eNOS/NO signal pathways may have contributed to the observed effects of NTG on EPCs. The mechanisms involved in the present findings need to be explored in future studies.

In conclusion, the results of the present study demonstrated that moderate doses of NTG induce NO production that contributes to the up-regulation of EPCs *in vitro*. Furthermore, excessive ONOO⁻ production and ROS-related mechanisms could be the main contributor to higher concentrations of NTG-induced EPC dysfunction. These findings not only provide insights into the complex cellular mechanisms of EPCs for impaired vascular repair, but also provide an opportunity for therapy development if future studies show that NTG contributes to proliferation activity of circulating EPCs in CAD patients.

Funding

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