NITROGLYCERIN ATTENUATES HUMAN ENDOTHELIAL PROGENITOR CELL DIFFERENTIATION, FUNCTION AND SURVIVAL

Jonathan M. DiFabio; George R. Thomas; Liana Zucco; Michael A. Kuliszewski; Brian M. Bennett; Michael J. Kutryk; John D. Parker.

Division of Cardiology, Department of Medicine, Mount Sinai and University Health Network Hospitals, and Department of Pharmacology, University of Toronto, Toronto, Canada (J.M.D., G.R.T., J.D.P.); Division of Cardiology, Terrence Donnelly Heart Centre, St. Michael’s Hospital, University of Toronto, Toronto, Canada (L.Z., M.A.K., M.J.K.); Department of Pharmacology and Toxicology, Faculty of Health Sciences, Queen’s University at Kingston, Kingston, Canada (B.M.B.)
Nitroglycerin Impairs Human Endothelial Progenitor Cells

John D. Parker, MD, FRCP(C), Division of Cardiology, Department of Medicine, Mount Sinai Hospital, 600 University Ave, Suite 1609, Toronto, Ontario, Canada M5G 1X5.

Tel: 416 586 4794 Fax: 416 586 8413 Email: jdp@ca.inter.net

Number of Text Pages: 20
Number of Tables: 1
Number of Figures: 5
Number of References: 38
Number of words in Abstract: 249
Number of words in Introduction: 440
Number of words in Discussion: 1286
Non-Standard Abbreviations: EPC (endothelial progenitor cell); EC (endothelial cell); GTN (nitroglycerin, glyceryl trinitrate); HSC (hematopoietic stem cell); VEGFR-2 (vascular endothelial growth factor receptor 2)
Section: Cardiovascular or Toxicology
Abstract:

Endothelial progenitor cells (EPCs) participate in angiogenesis and the response to chronic ischemia. Risk factors and cardiovascular disease attenuate EPC number, function and survival. Continuous therapy with nitroglycerin (glyceryl trinitrate; GTN) is associated with increased vascular oxidative stress, leading to nitrate tolerance and endothelial dysfunction. Thus, GTN therapy may also impact EPCs. The purpose of this study was to determine whether continuous exposure to GTN in vivo, or during ex vivo expansion, affects the circulating number and functional characteristics of human EPCs. To determine the effects of continuous in vivo GTN exposure, EPCs isolated from 28 healthy males before and after receiving 0.6 mg/hr GTN (n=17) or no treatment (n=11) for one week, were expanded for 6 days and compared. To determine the effects of continuous ex vivo GTN exposure, EPCs isolated before randomization were expanded for 6 days in medium supplemented with 100 nmol/L, 300 nmol/L or 1 µmol/L GTN. EPCs expanded without GTN served as controls (n=10). In vivo, GTN exposure significantly increased the percentage of circulating cells expressing the EPC marker CD34, and increased the susceptibility of expanded EPCs to apoptosis, but had no impact on the phenotypic differentiation or migration of EPCs. Ex vivo, GTN exposure increased apoptosis, while decreasing phenotypic differentiation, migration, and mitochondrial dehydrogenase activity of EPCs, as compared to EPCs expanded in the absence of GTN. Taken together, these results suggest that continuous GTN therapy might impair EPC-mediated processes, an effect that could be detrimental in the setting of ischemic cardiovascular disease.
Bone marrow–derived endothelial progenitor cells (EPCs) are thought to differentiate into functional endothelial cells (ECs), and participate in endothelial repair and the process of adult neovascularization (Szmitko et al., 2003; Asahara et al., 1997; Urbich and Dimmeler, 2004; Takahashi et al., 1999; Crosby et al., 2000). The results of a recent study indicate that EPCs may contribute up to 25% of ECs in newly formed blood vessels (Murayama et al., 2002). In addition, transplantation of EPCs into patients has been shown to induce blood flow recovery in ischemic limbs (Tateishi-Yuyama et al., 2002) and to improve myocardial viability after infarction (Assmsu et al., 2002). Thus, EPCs may be integral mediators of the response to chronic ischemia and acute myocardial infarction.

Research has also highlighted the potential of EPCs to serve as diagnostic and prognostic indicators. The number and migratory capacity of circulating EPCs has been shown to inversely correlate with risk factors for coronary artery disease (Vasa et al., 2001). Conversely, levels of circulating EPCs increase acutely after myocardial infarction (Massa et al., 2005; Shintani et al., 2001). Thus, the number and functional capacity of circulating EPCs may serve as a novel biological indicator of vascular health and cumulative cardiovascular risk (Hill et al., 2003; Hunting et al., 2005).

Both animal and human studies have confirmed that continuous therapy with nitroglycerin (glyceryl trinitrate; GTN), frequently employed in the treatment of coronary artery disease and congestive heart failure, increases the vascular levels of reactive oxygen species (ROS), such as superoxide anion (O2·−) and peroxynitrite (ONOO−), and causes endothelial dysfunction (Gori and Parker, 2002a; Gori and Parker 2002b). Increased vascular ROS, a risk factor implicated in the pathogenesis and progression of
several cardiovascular diseases, is considered to be a principal cause of GTN tolerance and nitrate-induced endothelial dysfunction (Gori and Parker, 2002b). However, the etiology of nitrate tolerance and nitrate-induced endothelial dysfunction is complex and certainly multifactorial, involving biochemical and enzymatic mechanisms within endothelial and vascular smooth muscle cells as well as neurohormonal adaptations and abnormalities in the autonomic nervous system (Gori and Parker, 2002a; Gori and Parker, 2002b), effects which may also have pathophysiological implications.

When we consider the adverse vascular effects of continuous GTN therapy, and the association between EPCs and vascular health, it follows that GTN therapy may negatively impact these cells. Thus, we sought to determine the effect of in vivo GTN exposure on the circulating number, phenotypic differentiation, migration and apoptosis of human EPCs. We also sought to determine whether ex vivo exposure to clinically relevant concentrations of GTN (simulating high-dose continuous therapy), would affect the phenotypic differentiation, migration, mitochondrial viability and apoptosis of human EPCs.

Materials and Methods:

Study Population

Twenty-eight healthy, non-smoking, male volunteers (18 to 35 years old) were enrolled. All subjects were instructed to abstain from alcohol or any drugs, including supplemental vitamins, for the duration of the study.
Study Protocol

This randomized, investigator-blind study was approved by the Mount Sinai Hospital and St. Michael’s Hospital ethics review committees. Written, informed consent was obtained from all subjects. After consent was obtained, standing heart rate and blood pressure were determined in triplicate. Subsequently, an 80 ml sample of peripheral venous blood was obtained. These pre-randomization (day 0) blood samples were used as a source of control EPCs. Subjects were then randomized in an investigator-blind manner to receive either transdermal administration of 0.6 mg/hr GTN given continuously (Transderm-Nitro, Novartis) or no treatment for 7 days (GTN treated n=17; non-treated n=11; ~3:2 ratio of GTN treated to non-treated subjects). A research nurse not involved in experimentation or analysis of the data conducted the randomization and the investigators remained blinded throughout the study and analysis period. On day 7, standing heart rate and blood pressure were determined and another 80 ml sample of venous blood was obtained. The number and functional characteristics of EPCs isolated and expanded from day 0 and day 7 blood samples of GTN treated subjects were compared in an effort to determine the effects of in vivo GTN exposure. EPCs isolated and expanded from the day 0 and day 7 blood samples of subjects randomized to receive no treatment acted as time controls. Ten day 0 blood samples were also used to investigate the effects of ex vivo GTN exposure on EPCs (see Ex Vivo GTN Exposure Protocol).

EPC Isolation and Expansion

EPCs were isolated and expanded by the enriched-medium method as described previously (Asahara et al., 1997). Briefly, the total mononuclear cell (MNC) fraction was
isolated from peripheral venous blood by Ficoll-Paque density gradient (Becton Dickinson) centrifugation. MNCs were washed twice with sterile phosphate-buffered saline (PBS; Sigma), and plated at a density of 1.5 x 10^6 cells/cm^2 on fibronectin-coated culture slides (Becton Dickinson) in endothelial cell basal medium-2 (EBM-2; Clonetics) supplemented with 5% fetal bovine serum, human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid (EGM-2-MV-SingleQuots; Clonetics). EPCs were expanded for 6 days at 37 ºC and 5% CO₂, with culture media changes every 24 hours.

**Ex Vivo GTN Exposure Protocol**

EPCs isolated from day 0 blood samples were expanded in medium supplemented with 100 nmol/L, 300 nmol/L or 1 µmol/L GTN (5 mg/ml GTN solution; Sabex). EPCs expanded without GTN served as controls (n=10). The EPCs were expanded for 6 days, with culture media changes and GTN supplementation every 24 hours. The phenotypic differentiation, apoptosis, migration and mitochondrial dehydrogenase activity of the EPCs were then determined.

**Quantification of Circulating EPCs**

Fluorescence activated cell sorting (FACS) was used to quantify the percentage of cells expressing EPC markers in the MNC fractions isolated from day 0 and day 7 blood samples. Briefly, the whole MNC fraction was isolated from peripheral venous blood, and re-suspended in PBS staining buffer to obtain a concentration of 1.5x10^6 cells/ml. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 (Becton
Dickinson) and phycoerythrin (PE)-conjugated mouse anti-human CD133 (Sigma) were used separately and in combination to label the cells. Unstained cells acted as controls. CD34 is an antigen expressed by late hematopoietic stem cells (HSCs; the putative precursor for EPCs), EPCs, and at a relatively low level on mature ECs (Szmitko et al., 2003; Asahara et al., 1997; Urbich and Dimmeler, 2004). CD133, also known as prominin or AC133, is expressed by early hematopoietic progenitor cells, but is absent on late EPCs and mature ECs (Szmitko et al., 2003; Urbich and Dimmeler, 2004). Cells were analyzed with a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 20,000 cells was quantified for each sample. The data was reported as the percentage of peripheral blood MNCs that expressed CD34 (MNC\(^{34+}\)), CD133 (MNC\(^{133+}\)) and both markers (MNC\(^{34/133+}\)).

**EPC Phenotypic Differentiation**

EPC phenotypic differentiation was evaluated with immunohistochemistry. After 6 days of expansion with or without GTN, EPCs were fixed with 2% paraformaldehyde (Sigma) in PBS for 10 minutes, washed three times with PBS, and stained with the mature endothelial cell markers rabbit anti-human vascular endothelial growth factor receptor-2 (VEGFR-2; Alpha Diagnostics) and FITC-conjugated EC-lectin (Ulex Europaeus Uea-1; Sigma). The binding of primary anti-VEGFR-2 antibody was confirmed by exposure of the cells to a FITC-conjugated secondary anti-rabbit antibody. Cells were mounted in VectaShield mounting medium (Vector Laboratories) with propidium iodide as a nuclear marker. Cells stained with only propidium iodide served as negative controls. EPCs were then visualized with dual-emission confocal microscopy, and the percentage of cells
binding EC-lectin or expressing VEGFR-2 in four random microscopic fields (X 200 magnification) was determined.

**EPC Apoptosis Assay**

EPC apoptosis was quantified with terminal deoxyuridine triphosphate nick end-labelling (TUNEL), a staining method used to detect DNA fragmentation. After 6 days of expansion, EPCs were fixed with 2% paraformaldehyde in PBS for 10 minutes and then washed three times with PBS. EPCs were then permeabilized with 0.2% Triton-X 100 and subjected to DeadEnd Fluorometric TUNEL System staining (Promega) according to the manufacturer’s recommended protocol. EPCs were mounted in VectaShield mounting medium with propidium iodide as a nuclear marker. Cells were visualized with dual-emission confocal microscopy and the percentage of apoptotic cells in four random microscopic fields (X 200 magnification) was determined.

**EPC Mitochondrial Dehydrogenase Activity Assay**

EPC mitochondrial dehydrogenase activity was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell viability. After being expanded for 6 days, EPCs were detached from the fibronectin coated slides with 0.25% trypsin, then counted, and suspended in serum-free medium at a concentration of 5 x 10^5 cells/ml in a 96-well culture plate (200 µl/well). 10 µl MTT (5 g/l) was added to each well and the preparation was incubated for 2 h at 37 °C.
Following the incubation, the EPC preparation was agitated with 200 µl of MTT solubilization solution (10% Triton X with 0.1 N HCl in anhydrous isopropanol) for 10 minutes before the optical density was measured at 490 nm and 650 nm with an ultraviolet plate reader. Each experiment was performed in quadruplicate, with serum-free medium acting as a control. The data is presented as the optical density at 490 nm minus the optical density at 650 nm, with the values being directly proportional to the level of mitochondrial dehydrogenase activity.

**EPC Migration Assay**

EPC migration was evaluated with the modified Boyden’s chamber assay. After 6 days of expansion, EPCs were detached from the fibronectin coated slides with 0.25% trypsin, then counted and suspended in serum-free M199 culture medium (Sigma) to obtain a concentration of 5 x 10⁵ cells/ml. 500 µl of the EPC suspension was placed in the upper compartment of a modified Boyden’s chamber (Becton Dickinson) and human VEGF (15 ng/ml) in serum-free M199 media was placed in the lower compartment. A membrane with 8 µm pores separated the upper and lower compartments. After 5 h incubation at 37 °C, the membrane was washed gently with PBS to remove any non-migratory cells, and fixed with 50% methanol. EPCs trapped in the pores while migrating into the lower compartment were stained with Diff-Quick staining solutions (Dade Behring) and visualized with an inverted light microscope. Experiments conducted without VEGF in the lower compartment served as negative controls. Data is presented as the total number of cells counted in five random microscopic fields (X 200 magnification).
GTN Metabolism Assay

To determine whether EPCs can metabolize GTN to form the metabolites 1,2- and 1,3-glyceryl dinitrate (GDN), EPCs expanded for 6 days under control conditions were detached from the fibronectin coated slides with 0.25% trypsin, counted and suspended in endothelial growth medium at a concentration of 5 x 10^5 cells/ml. The EPC suspension was then incubated with 100 nmol/L GTN for 24 hours at 37 °C. Media supplemented with 100 nmol/L GTN acted as controls. After the incubation, the preparations were immediately centrifuged to remove the cells, and the medium was collected. 1,2- and 1,3-GDN, and GTN were then extracted from the medium and a previously described method utilizing megabore capillary column gas liquid chromatography (McDonald and Bennett, 1990) was used to quantify the amounts of these metabolites present (n=6). Data is presented as the ratio of the total concentration of GDN’s (1,2- and 1,3-GDN) to GTN.

Vehicle Exposure Protocol

To determine the contribution of vehicle to the observed effects of GTN on EPCs, cells isolated from 6 day 0 blood samples were expanded in medium supplemented daily with propylene glycol and ethanol at concentrations equal to those encountered in cultures supplemented with 1 µM GTN, the highest level of exposure employed. The vehicle solution was 29% propylene glycol, and 28.5% ethanol in sterile, deionized water. EPCs expanded without vehicle served as controls. After 6 days of expansion with or without vehicle, EPCs were assayed for phenotypic differentiation, as determined by EC-lectin binding and VEGFR-2 expression, and apoptosis, as determined by TUNEL.
Statistical Analysis

For the in vivo protocol, within group comparisons were made using Student’s t-test for paired data. Comparisons between GTN-treated and control subjects were made using Student’s t-tests for unpaired data. Where data was not normally distributed, nonparametric testing was employed using the Wilcoxon Signed Rank test. For the ex vivo protocol, the effect of different concentrations of GTN on EPCs was compared using repeated measures analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test. Data are presented as mean ± standard error of the mean (SEM).

Results:

In Vivo GTN Exposure

The percentages of circulating MNC$^{34+}$, MNC$^{133+}$ and MNC$^{34/133+}$ in day 0 and day 7 blood samples were determined with FACS (Figure 1A and Table 1A). The percentage of circulating MNC$^{34+}$ increased ~ 2 fold with in vivo GTN exposure (Figure 1A and Table 1A; $P=0.04$). The percentage of MNC$^{133+}$ and MNC$^{34/133+}$ was not significantly altered due to in vivo GTN exposure (Table 1A, $P=NS$). FACS analysis of day 0 and day 7 MNC fractions isolated from non-treated subjects was conducted to establish the number of circulating EPCs, and the stability of these numbers over 7 days in normals receiving no treatment. The percentage of MNC$^{34+}$, MNC$^{133+}$ and MNC$^{34/133+}$ at day 0 and day 7 was not significantly different in subjects who received no treatment (Table 1A, $P=NS$). To evaluate EPC apoptosis, EPCs expanded from day 0 and day 7 blood samples were subjected to TUNEL (Figure 1B). The percentage of apoptotic cells was significantly
increased in EPCs expanded from subjects who received GTN (Day 0: 6 ± 1% versus Day 7: 17 ± 4%; \( P<0.05 \)), but was unaltered in EPCs expanded from subjects who received no treatment (Day 0: 5 ± 1% versus Day 7: 5 ± 1%; \( P=\text{NS} \)). The modified Boyden’s chamber assay was used to assess the \textit{in vitro} migratory capacity of EPCs expanded from day 0 and day 7 blood samples (Table 1B). The capacity for migration was not significantly different between EPCs expanded from day 0 and day 7 blood samples of subjects who received GTN, or no treatment (\( P=\text{NS} \) for both). The phenotypic differentiation of MNCs to EPCs, as indicated by EC-lectin binding, and VEGFR-2 expression, was assessed after expansion (Table 1C). The percentage of cells binding EC-lectin, and expressing VEGFR-2 was not significantly different between cells expanded from day 0 and day 7 blood samples of subjects who received GTN (\( P=\text{NS} \) for all groups) or no treatment (\( P=\text{NS} \) for all groups).

\textbf{Ex Vivo GTN Exposure}

After 6 days of expansion under control conditions, 5 ± 1% of adherent cells stained positive for apoptosis with TUNEL (Figure 2), 92 ± 1% bound EC-lectin (Figure 3A), and 92 ± 2% expressed VEGFR-2 (Figure 3B). Apoptosis was significantly increased in cells expanded with 100 nmol/L, 300 nmol/L and 1 \( \mu \)mol/L GTN (Figure 2, 12 ± 2%, 15 ± 2% and 17 ± 1% respectively, \( P<0.001 \) ANOVA, Student-Newman-Keuls post hoc results: 100 nmol/L vs. 300 nmol/L \( P<0.05 \), 300 nmol/L vs. 1 \( \mu \)mol/L \( P=\text{NS} \)).

Representative photomicrographs of TUNEL staining are presented below the graph in Figure 2. Note the increased yellow fluorescence resulting from dual staining with the red fluorescing nuclear label propidium iodide and green fluorescing TUNEL, indicating
apoptosis, in cells expanded with GTN. EC-lectin binding was significantly decreased in adherent cells expanded with 100 nmol/L, 300 nmol/L and 1 µmol/L GTN (Figure 3A, 75 ± 2 %, 67 ± 1 % and 56 ± 3 % respectively, P<0.001 ANOVA, Student-Newman-Keuls post hoc results: 100 nmol/L vs. 300 nmol/L P<0.05, 300 nmol/L vs. 1 µmol/L P<0.05). VEGFR-2 expression was also significantly decreased with all treatments compared to control cells (Figure 3B, 77 ± 2 %, 65 ± 5 % and 57 ± 4 % respectively, P<0.001 ANOVA, Student-Newman-Keuls post hoc results: 100 nmol/L vs. 300 nmol/L P<0.05, 300 nmol/L vs. 1 µmol/L P<0.05). Representative photomicrographs of EC-lectin binding and VEGFR-2 staining are presented below the corresponding graphs in Figure 3. Note the decreased yellow fluorescence resulting from dual staining with the red fluorescing nuclear marker propidium iodide and green fluorescing FITC, which indicates either EC-lectin binding or VEGFR-2 expression, in cells expanded with GTN. EPCs expanded with 100 nmol/L and 1 µmol/L GTN demonstrated a significantly decreased capacity for in vitro migration as compared to control EPCs (Figure 4, Control, 92 ± 7 cells migrated; 100 nmol/L GTN, 73 ± 9; 1 µmol/L GTN, 56 ± 10; P<0.001 ANOVA, Student-Newman-Keuls post hoc results: 100 nmol/L vs. 1 µmol/L P<0.05). The effect of continuous ex vivo GTN exposure on EPC mitochondrial dehydrogenase activity was determined with the MTT cell proliferation assay (Figure 5). Mitochondrial dehydrogenase activity was significantly reduced in EPCs expanded with 100 nmol/L and 1 µmol/L GTN as compared to controls (Control, 0.086 ± 0.005; 100 nmol/L, 0.075 ± 0.003; 1 µmol/L, 0.066 ± 0.006; P=0.002 ANOVA, Student-Newman-Keuls post hoc results: 100 nmol/L vs. 1 µmol/L P<0.05). To exclude a contribution of GTN’s vehicle to the observed effects, we expanded EPCs with propylene glycol and ethanol at the
concentrations encountered in cultures supplemented with 1 µmol/L GTN, the highest level of exposure employed. Exposure to the vehicle of GTN during the 6 day expansion did not significantly alter EPC apoptosis, as determined by TUNEL (Control, 4 ± 1 % versus Vehicle, 4 ± 1 %; \( P=\text{NS} \)), or phenotypic differentiation, as determined by EC-lectin binding (Control, 88 ± 2 % versus Vehicle, 86 ± 3 %; \( P=\text{NS} \)) and VEGFR-2 expression (Control, 87 ± 2 % versus Vehicle, 86 ± 4 %; \( P=\text{NS} \)).

**EPCs and GTN Biotransformation**

Although we observed low-level non-cellular biotransformation of GTN due to serum components in the control incubations, 1,2- and 1,3-GDN formation doubled in the presence of EPCs (ratio of GDN’s to GTN after incubation: control 1.2 ± 0.07; EPCs 2.2 ± 0.09; \( P<0.05 \)) indicating that these cells can metabolize GTN.

**Discussion:**

In the present study, we demonstrated that both *in vivo* and *ex vivo* exposure to GTN had effects on human EPCs. Continuous *ex vivo* exposure dose-dependently inhibited the phenotypic differentiation of MNCs to EPCs, as indicated by decreased binding of EC-lectin and decreased expression of the vital growth factor receptor VEGFR-2 after 6 days of expansion. We also observed a significant increase in the level of apoptosis in EPCs expanded with GTN. This result is in accord with previous studies demonstrating GTN-induced apoptosis of Jurkat leukemic cells (Ushmorov et al., 1999) and several human colon cancer cells lines (Millet et al., 2002), although these studies employed millimolar concentrations of GTN. EPCs expanded with GTN also displayed
decreased migration in response to a concentration gradient of VEGF, a potent mediator of EPC mobilization and migration to sites of vascular damage and/or neovascularization (Szmitko et al., 2003). The decreased migration of EPCs expanded with GTN may be directly attributable to the observed decrease in VEGFR-2 expression, as this receptor is required for VEGF-mediated migration (Szmitko et al., 2003; Urbich and Dimmeler, 2004). Finally, we observed decreased mitochondrial dehydrogenase activity in EPCs expanded with GTN, a finding that would suggest a GTN-induced mitochondrial insult and decreased cellular viability. Exposure to the vehicle of GTN caused no significant change in the extent of phenotypic differentiation, or level of apoptosis as compared to cells expanded under control conditions, suggesting that GTN was the mediator of the observed effects. We also demonstrated that these cells metabolize GTN, as EPCs produced roughly twice the 1,2- and 1,3-GDN from 100 nmol/L GTN as compared to control incubations with growth medium alone. This result further implicates GTN as the mediator of the effects we observed, as the metabolism of GTN is thought to be integral to the development of tolerance, nitrate-induced endothelial dysfunction, and other documented detrimental biochemical effects of this compound (Sydow et al., 2004).

The concentrations of GTN we employed in the \textit{ex vivo} GTN exposure experiments are approaching the upper limit of what would be achieved during therapy, as administration of GTN in humans via sublingual, transdermal or intravenous routes, generally yields plasma concentrations in the low nanomolar range (Hashimoto and Kobayashi, 2003; Curry et al., 1984; Imhof et al., 1982; Armstrong et al., 1979; Armstrong et al., 1980; Jewell et al., 1992). Nevertheless, we believe that the concentrations employed have clinical relevance.
GTN yielded a maximum venous plasma concentration of 45 nM in 119 healthy volunteers (Curry et al., 1984), and studies have documented that plasma concentrations exceeding 100 nM can be achieved with moderate intravenous infusion rates of GTN (Imhof et al., 1982; Curry et al., 1993; Booth et al., 1994). Thus, plasma concentrations approaching or exceeding 100 nM are achieved during GTN therapy. Furthermore, reported plasma concentrations of GTN must be interpreted with care, as the pharmacokinetics of this compound are characterized by prominent interindividual variability and a marked arteriovenous concentration gradient, with higher concentrations of GTN in arterial plasma (Armstrong et al., 1982). The majority of human studies reporting plasma concentrations of GTN either did not specify the sampling site or used venous plasma, in which case the reported plasma concentrations would have been substantially lower than that found in arterial plasma. Given this background, the concentrations of GTN we employed are clinically relevant.

To establish whether these same effects would result from in vivo exposure to GTN, we exposed healthy, male subjects to 0.6 mg/hr GTN for 7 days. In contrast to the results from our ex vivo GTN exposure protocol, in vivo exposure to GTN did not convey the same effects on EPC phenotypic differentiation or migration. However, EPCs isolated from subjects who received GTN displayed a significantly increased level of apoptosis after 6 days of expansion. Despite the observed increase in apoptosis of EPCs, there was a significant increase in the percentage of circulating MNC34+, a finding which suggests that HSC/EPC production/mobilization may have increased in subjects receiving GTN to compensate for an increased rate of EPC turnover. Importantly, studies have documented an increase of similar magnitude in the number of circulating MNC34+ in humans after
acute myocardial infarction (Massa et al., 2005; Shintani et al., 2001), and in the early phase of congestive heart failure (Valgimigli et al., 2004). Conversely, the number of circulating MNC34+ has been shown to be reduced in patients with risk factors for coronary artery disease (Vasa et al., 2001). Therefore, it is clear that a number of factors are involved in the regulation of both the production and utilization of these precursors, and that any change in the circulating number of these cells can represent a change in their production rate or utilization rate or both. Importantly, CD34 positive ECs circulate within peripheral blood for a short time after being shed from the vessel wall (Urbich and Dimmeler, 2004), and thus we cannot exclude the potential contribution of these cells to the observed increase in circulating MNC34+. Overall, the results of the in vivo exposure protocol indicate that continuous exposure to GTN modifies the number of circulating MNC34+ and increases the susceptibility of EPCs to apoptosis ex vivo. However, the clinical significance of these effects remains to be determined. Regardless, the observed increase in EPC apoptosis is worrisome, and augments the growing body of evidence that long-term, continuous GTN therapy may be harmful.

This was an observational study and we did not attempt to elucidate the underlying mechanisms of the observed effects. However, it may be that a GTN-induced increase in the production of O2- and ONOO- (Munzel et al., 1995), and/or inhibition of the mitochondrial electron transport chain (Ushmorov et al., 1999; Millet et al., 2002; Sydow et al., 2004; Chen et al., 2002; Boime and Hunter, 1971), may have contributed to the observed effects. Endothelial nitric oxide synthase (eNOS) activity is thought to be integral to HSC/EPC function (Murohara et al., 1998; Guthrie et al., 2005), and thus, uncoupling/inhibition of eNOS, an effect documented to occur with prolonged GTN
exposure (Munzel et al., 2000; Kaesemeyer et al., 2000) may have played a role in the observed effects. Finally, GTN-induced impairments of endothelial NO-signal transduction pathways (Mulsch et al., 2001; Warnholtz et al., 2002) may have also contributed to the observed effects of GTN on EPCs. The mechanisms described above are thought to contribute significantly to the development of nitrate tolerance and nitrate-induced endothelial dysfunction, both documented to occur within 24-48 hours of continuous exposure to GTN (Gori and Parker, 2002a; Gori and Parker 2002b). Further investigation will be necessary to establish whether these mechanisms played a role in the observed effects.

In summary, *in vivo* exposure to 0.6 mg/hr GTN for 7 days significantly increased the percentage of circulating MNC34+, and the percentage of apoptotic EPCs after 6 days of expansion under control conditions. *Ex vivo* exposure of human EPCs to GTN dose-dependently attenuated phenotypic differentiation, migration and mitochondrial dehydrogenase activity, and was associated with increased EPC apoptosis. Given that no study has unequivocally proven that EPC dysfunction as detected in the laboratory correlates with impairments of EPC mediated endothelial repair and neovascularization in humans, we cannot conclude yet whether the observed effects would be harmful. Furthermore, this study was conducted in healthy volunteers, and thus, these effects may not be observed in cardiovascular disease states. Nonetheless, these data augment the growing body of evidence that continuous GTN therapy has systemic effects unrelated to its mode of action, and illuminate a novel mechanism by which this compound may exert negative cardiovascular effects. With accumulating evidence of nitrate-induced
cardiovascular toxicity, the authors believe it is time to organize large-scale clinical trials to establish the effects of continuous nitrate therapy on long-term clinical outcome.

Acknowledgements:

The authors would like to thank the members of the Clinical Cardiovascular Research Laboratory at Mount Sinai Hospital for their assistance in completing this study.
References:


Footnotes:

This study was funded by an operating grant from the Canadian Institutes for Health Research (J.D.P.), the Heart and Stroke Foundation of Ontario (Grant T 5162 to B.M.B.; Career Investigator Award to J.D.P.), University of Toronto Fellowships (J.M.D.) and an Ontario Graduate Scholarship (J.M.D.).

Please address reprint requests to John D. Parker, Mount Sinai Hospital, 600 University Ave, Suite 1609, Toronto, Ontario, Canada M5G 1X5. Email: jdp@ca.inter.net
Figure Legends:

Figure 1. A, Percentage of MNC<sup>34+</sup> in day 0 and day 7 peripheral blood samples of subjects who received no treatment or GTN. The percentage of MNC<sup>34+</sup> was significantly increased due to <i>in vivo</i> GTN exposure. B, Percentage of total cells expanded from day 0 and day 7 blood samples of control and GTN-exposed subjects that were TUNEL positive (% Apoptosis) after 6 days of expansion. EPCs expanded from day 7 blood samples of GTN-exposed subjects displayed a significantly increased level of apoptosis. Each value represents the mean ± SEM.

Figure 2. Percentage of total cells that were TUNEL positive (% Apoptosis) after 6 days of expansion with 100 nmol/L, 300 nmol/L, 1 µmol/L or no GTN. <i>Ex vivo</i> GTN exposure significantly increased the percentage of apoptotic EPCs as compared to cells expanded without GTN (*<i>P</i>&lt;0.05 versus control; †<i>P</i>&lt;0.05 versus 300 nM). Each value represents the mean ± SEM (n=8). Red fluorescence indicates the nucleus. Green fluorescence indicates TUNEL staining. Note the increased dual staining (yellow fluorescence; apoptosis) in GTN exposed EPCs (X 200 magnification).

Figure 3. Percentage of total cells A, binding EC-lectin and B, expressing VEGFR-2 after 6 days of expansion with 100 nmol/L, 300 nmol/L, 1 µmol/L or no GTN. <i>Ex vivo</i> GTN exposure dose-dependently decreased the binding of EC-lectin, and expression of VEGFR-2 as compared to cells expanded without GTN (*<i>P</i>&lt;0.05 versus control; †<i>P</i>&lt;0.05 versus 300 nmol/L; # <i>P</i>&lt;0.05 versus 1000 nmol/L). Each value represents the mean ± SEM (n=8). Red fluorescence indicates the nucleus. Green fluorescence indicates
either EC-lectin binding or VEGFR-2 expression. Note the decreased dual staining (yellow fluorescence) in GTN exposed cells (X 200 magnification).

Figure 4. Migration of EPCs expanded with 100 nmol/L, 1 µmol/L or no GTN for 6 days. Ex vivo GTN exposure dose-dependently decreased the migratory capacity of EPCs as compared to those expanded without GTN (*$P<0.05$ versus control; †$P<0.05$ versus 1000 nmol/L). Each value represents the mean of the total number of cells migrated ± SEM (n=10). The results of migration assays conducted with control cells and no VEGF chemoattractant (negative controls) are also presented.

Figure 5. Mitochondrial dehydrogenase activity (OD 490 nm – OD 650 nm) of EPCs expanded with 100 nmol/L, 1 µmol/L or no GTN for 6 days. Ex vivo GTN exposure dose-dependently decreased the mitochondrial dehydrogenase activity of EPCs as compared to those expanded without GTN (*$P<0.05$ versus control; †$P<0.05$ versus 1000 nmol/L). Each value represents the mean ± SEM (n=6).
Table 1. A, Percentage of circulating MNC$^{34+}$, MNC$^{133+}$ and MNC$^{34/133+}$. B, Migration of EPCs. C, Phenotypic differentiation of MNCs to EPCs.

<table>
<thead>
<tr>
<th></th>
<th>Control Day 0</th>
<th>Control Day 7</th>
<th>GTN Day 0</th>
<th>GTN Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating EPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% MNC$^{34+}$</td>
<td>0.48±0.08</td>
<td>0.56±0.13</td>
<td>0.46±0.18</td>
<td>0.86±0.35*</td>
</tr>
<tr>
<td></td>
<td>[0.46]</td>
<td>[0.4]</td>
<td>[0.32]</td>
<td>[0.4]</td>
</tr>
<tr>
<td>% MNC$^{133+}$</td>
<td>0.24±0.05</td>
<td>0.17±0.02</td>
<td>0.14±0.02</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td></td>
<td>[0.26]</td>
<td>[0.18]</td>
<td>[0.15]</td>
<td>[0.09]</td>
</tr>
<tr>
<td>% MNC$^{34/133+}$</td>
<td>0.22±0.03</td>
<td>0.15±0.03</td>
<td>0.12±0.01</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td></td>
<td>[0.23]</td>
<td>[0.14]</td>
<td>[0.11]</td>
<td>[0.11]</td>
</tr>
<tr>
<td>EPC Migration Assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Number of Cells</td>
<td>96±13 [78]</td>
<td>88±18 [57]</td>
<td>88±8 [94]</td>
<td>80±8 [76]</td>
</tr>
<tr>
<td>Migrated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Binding Lectin</td>
<td>92±1 [92]</td>
<td>88±2 [90]</td>
<td>89±1 [90]</td>
<td>84±4 [88]</td>
</tr>
<tr>
<td>Expressing VEGFR-2</td>
<td>88±3 [90]</td>
<td>84±4 [86]</td>
<td>89±2 [92]</td>
<td>83±4 [86]</td>
</tr>
</tbody>
</table>

Values are mean ± SEM [median]. * $P<0.05$ for GTN day 0 versus GTN day 7.
Fig. 1

A

% of MNC$^{34+}$ in peripheral blood

B

% Apoptosis

DAY 0  DAY 7  DAY 0  DAY 7

DAY 0  DAY 7  DAY 0  DAY 7

P=0.04  P=0.002

Control  GTN
Fig. 2

% Apoptosis

Concentration of GTN (nM)

0 100 300 1000

* †

*
Fig. 3B

% VEGFR-2 Positive

Concentration of GTN (nM)

0 100 300 1000

* †

* #

*
Fig. 4

The graph shows the number of cells migrated in response to different concentrations of GTN (nM). The concentrations tested were 0, 100, 1000, and NO VEGF. The graph indicates a significant increase in cell migration at 100 and 1000 nM compared to the control (0 nM) and NO VEGF conditions, as denoted by the asterisks (*) and double asterisks (†) on the bars.
Fig. 5

Concentration of GTN (nM)

(OD 490 nm - OD 650 nm)

0 100 1000

0 0.02 0.04 0.06 0.08 0.1

* * †