

Studying the Anti-aging Effect of Nitric Oxide on the Cell Proliferation and Telomerase Activity of Human Cord Blood Hematopoietic Stem Cells

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Abstract

Introduction: Accumulating evidences indicated that during increasing of the animals' age, the number and the functional properties of hematopoietic stem cells (HSCs) become altered because of a gradual decrease in replication potential. The efficient factors in this process are DNA damaging, reduced telomerase activity, shortening of telomeres and oxidative stresses. For overcoming these factors, using of the anti-oxidants and activating of telomerase would be effective. The aim of this research was to study the effect of Nitric Oxide (NO) as an anti-oxidant on the cell proliferation, cell viability and telomerase activity of HSCs *in vitro*.

Methods: HSCs were isolated from human cord bloods. Cells were treated by L-Arginine and Sodium Nitro-Pruside (as NO donors) in a dose dependent manner. The cell viability and proliferation were assayed by trypan blue, MTT and BrdU methods. The profile of aging was assayed by senescence sensitive β -Galactosidase staining and telomerase activity was assayed by TRAP-PCR ELISA method. Finally, Nitric Oxide Synthetase mRNA expression level was analyzed by RT-PCR.

Result: HSCs those treated with SNP exhibited an increase 3-7 fold (400-1000 μ mol) in NO production in comparison to untreated control cells (140 μ mol). Treatment of cells by L-Arg resulted lower release of NO (Up to 200 μ mol) in comparison to SNP. Increasing NO production resulted to the inducing of cells growth potential and proliferation parameters up to 40% which accompanied by the increasing of telomerase activity up to 25% in the presence of 100 μ M of SNP or 1.0 mM of L-Arg in comparison to untreated control cells.

Discussion: The present work demonstrates that NO affect telomerase activity and cellular replicative capacity in human hematopoietic stem cells. A significant behavior was observed on the telomerase activity and cell proliferation after treatment of cells. Induction of cell proliferation was accompanied by a slight inhibition of HSCs senescence. Finally the telomerase induction and reduction in cell senescence were accompanied by increasing of the cell proliferation parameters.

Key Words: Nitric Oxide, Hematopoietic Stem Cells, Anti-aging, Telomerase, Cell proliferation.

Introduction

Hematopoiesis is maintained throughout life by self-renewing stem cells with a high potential for proliferation and multi-lineage differentiation. Most circulating blood cells have a finite lifespan. To compensate for the continual loss of differentiated cells, it is estimated that an adult human needs to

produce between 10^{11} and 10^{12} mature blood cells per day.(1) In addition to normal cell turnover, the hematopoietic system must be able to respond rapidly to illness and trauma. Accumulating evidences indicated that as animals age, the number and the functional properties of hematopoietic stem cells (HSCs) become altered.(2) In spite of previous

hypothesis that stem cells have indefinite ability for self-renewal either *in vivo* or *in vitro* with the time passing, they show a decrease in their replication ability and with explanation that aging is a gradual decrease in replication potential.(3) The efficient factors in this process are DNA damaging, reduced telomerase activity, shortening of telomeres and oxidative stresses.(4, 5) In addition to such quantitative and functional changes of HSCs with aging, the density or activity of several cell-surface antigens and membrane transporters and enzymes were changed during ontogeny and throughout adulthood.(2)

Last decade researches have been made in understanding age related changes at two cellular loci; telomeres and the nucleolus.(6) The idea that shortening of telomeres could account for the gradual loss of replicative capacity or cellular senescence of cultured HSCs arose from the inability of DNA polymerase to fully replicate the ends of linear DNA duplexes.(7, 8) It was discovered that telomere length in HSCs decreases as a function of both ontogeny and age. Studies have shown striking differences in the mean length of telomeric repeat sequences at the end of chromosomes from HSCs at different stages of development and decrease of telomerase activity.(9, 10) How telomerase inactivation and telomeres shortening triggers cell senescence and whether it contribute to aging *in vivo* are under investigation.

Reactivation of telomerase in cultured human cells extends their replication life span beyond the Hay flick limit.(6) The activation of the telomerase that serves to maintain telomere length and preserve it is also thought to play an important role in the self-renewal of HSCs.(11, 12) Primary cell culture and genetic engineering systems have provided valuable experimental tools for investigation into the mechanism of aging and anti-aging studies at the cellular level *in vitro*.(13, 14) Therefore for overcoming these factors, using of telomerase activation and antioxidant could be prevented the aging of HSCs *in vitro*. Many antioxidants as anti-aging factors were studied and reviewed to prevent oxidative damage to cells.(15, 16)

The relation between reduced HSCs growth by aging process and telomerase activity was not clearly studied. The aim of this research was to study the effect of Nitric Oxide (NO) as an anti-oxidant on the cell proliferation, cell viability and telomerase activity of human HSCs *in vitro*. NO is an efficient physiologic factor in cellular metabolism and cell signaling in oxidative damages. NO is produced from the degradation of

L-Arginine in a reaction that is catalyzed by Nitric Oxide Synthetase (NOS, E.C. 1.14.13.39) and require several cofactors including calmodulin, oxygen, FAD, FMN, NADPH and BH₄.(17) NO is a highly reactive, diffusible, short-lived gaseous free radical and messenger molecule plays an important role in the regulation of a wide range of physiological and pathophysiological processes including: cellular immunity, neurotransmission, vasodilatation, angiogenesis, cardiomyogenesis, modulate hemangioblast activity of HSC, tumorocidal and microbiocidal activity.(18- 23)

By consideration the critical role of the telomerase in cell growth and cell proliferation of HSCs, the relation between telomerase activity and NO was not clearly studied. In another word, the molecular and cellular mechanisms of NO (as an anti-oxidant) in the cell growth and cell proliferation and the role of telomerase in these phenomena were not clearly understood. In this research, we hypothesized that treatment of HSCs by NO donors would up regulated telomerase activity, that associated with the increased cell proliferation and prevent the aging of the HSCs. For this purpose umbilical human cord blood HSCs were isolated and cultured. The cells were treated by L-Arginine and Sodium Nitro-Pruside as NO donors in a dose dependent manner. Then the cell proliferation, cell viability and telomerase activity were analyzed.

Materials and Methods

Cord blood collection

Umbilical Cord Bloods (CB) to be discarded were collected. CB was obtained from informed and consenting donors at the Mustafa Khomeini Hospital. The umbilical cord was clamped according to standard hospital procedure and CB collections were performed *ex utero*. CB was collected into a sterile collection 50 ml falcon tubes containing 10 ml of Citrate Phosphate Dextrose solution (Sigma, St. Louis, MO, USA). The collection volume varied between 15–40 ml. After fourfold dilution with Ca⁺⁺/Mg⁺⁺ free Hanks' Balanced Salt Solution (HBSS) (Sigma St. Louis, MO, USA), mono-nuclear cells (MNCs) were isolated by density gradient using 10 ml of Ficoll-Paque reagent (Nycomed, Oslo, Norway) and 30 ml of diluted CB. The two-phase system was centrifuged at 400×g for 40 minutes without break, at 20°C in a swinging-bucket rotor (Beckman Coulter Inc. CA, USA). MNCs, collected from the interface of the two phases, were washed twice with PBS. MNCs were counted by hemocytometer

neobar and cells viability was assayed by trypan blue dye exclusion test.

Separation of CD34⁺/CD133⁺ cells

Enrichment of hematopoietic stem cells in cord blood is based on the expression of certain surface antigens, such as CD34 and CD133, or on the lack of expression of lineage-specific antigens. Immunomagnetic negative selection kit (StemSep StemCell Technologies, Vancouver, Canada) was used for the isolation of CD34⁺ or CD133⁺ cells by using paramagnetic microbeads conjugated to specific monoclonal antibodies. In negative selection of lineage-negative (Lin⁻) cells, the unwanted cells are labeled with antibodies against known markers for mature hematopoietic cells (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A) and retained in the column. Unlabeled cells pass through the column and are collected as the Lin⁻ cell fraction. To enrich progenitor cells, lineage committed cells were depleted.(24, 25)

The conditions resulting in optimal separation of Lin⁻ cells were 100 µl of Progenitor Enrichment Cocktail and 60 µl of magnetic iron particles per 8 ×10⁷/ml MNCs, as recommended by the manufacturer. MNCs (8 ×10⁷/ml) were labeled with Progenitor Enrichment Cocktail (100 µl/ml) containing antibodies against CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and Glycophorin A (StemCell Technologies, Vancouver, Canada) at room temperature for 15 minutes. Subsequently, the cell suspension was incubated with magnetic iron particles (60µl/ml) at room temperature for 15 minutes. Cell suspension was loaded into MACS LD column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and unlabeled cells passing through the column were collected (Lin⁻ fraction). The column was then washed twice with 1 ml of buffer and the remaining Lin⁺ cells were collected for control purposes.

Cell numbers and controls of cell viability were determined by a hemocytometer, using the Trypan blue test.

For suspension culture and various assays, isolated cells were cultured in RPMI-1640 medium (Gibco, Paisley, U.K.) that supplemented by FCS 20% with combination of the following cytokines; recombinant human erythropoietin (rhEPO) 6 U/ml (Roche, Mannheim, Germany), recombinant human interleukine-3 (rhIL-3) 50 ng/ml (Sandoze, Basel. Switzerland), recombinant human Granulocyte-Macrophage colony stimulating factor (rhGM-CSF)

20 ng/ml (Sandoze, Basel. Switzerland) and rhIL-6 20 ng/ml (Sandoze, Basel. Switzerland).

Treatment of cells by nitric oxide donors and measurement of the generation of nitric oxide

The amount of NO generation from L-Arginine and Sodium Nitro-Prosides (SNP) in our system was determined. Cord blood HSCs were treated by 0-10 mM of L-Arginine (Merck Darmstadt, Germany) or 0-500 µM of SNP (Merck, Darmstadt, Germany) in RPMI-1640 medium, supplemented by 20% of FCS, rhIL-3, rhGM-CSF, rhEPO and rhIL-6 for up to 120 hr at 37°C.

In some experiments L-Arginine or SNP were added to RPMI medium alone (cell free system) in above condition as controls. Cell-free supernatants were recovered after incubation. Produced NO was quantitatively measured as NO₃⁻ (Nitrate: a stable metabolite of NO) and NO₂⁻ (Nitrite) concentrations by using enzymatic and colorimetric NO assays according the methods described by Schmidt (1996) and Granger et al (1999).(26, 27) In this method, we used reduction of nitrate by commercially *Aspergillus niger* nitrate reductase and measurement of the product (Nitrite) by Griess reagent in micro titer plate. In brief, 50 µl of cells condition medium or standards were added to each well. Just prior to each assay freshly made solutions of NADPH (0.02 M) (Sigma, St. Louis, MO, USA) 10µl, mixed solution of Glucose-6- Phosphate 50mM (Sigma, St. Louis, MO, USA) and Glucose-6-Phosphate Dehydrogenase (100 U/ml) 23 µl (Sigma, St. Louis, MO, USA), Nitrate Reductase 0.1 U/ml, 10 µl (Sigma, St. Louis, MO, USA) and 7µl of Tris buffer 1.0 M pH 7.5 were prepared separate and added to related wells.

Reactant were mixed finely and incubated at room temperature for 30 min. After that 100µl of freshly prepared Greiss reagent, (1% Sulfanilamide (Sigma, St. Louis, MO, USA) and 0.1% Naphtylenediamine(Sigma, St. Louis, MO, USA) in 2.8% of ortho-phosphoric acid) was added to each well and incubated at room temperature for 10 min. Finally the absorbance were measured at 550nm by ELISA reader (Labsystems Multiskan, Roden, Netherlands).

Cell growth and Proliferation assays

In order to examine the effect of Nitric Oxide on the cell growth and cell proliferation of the HSCs, cells were collected after 120 hr and the total cell number, viability, Bromo deoxy-Uridine incorporation (BrdU) and MTT cell proliferation assays were done as below. Cell number and

viability was enumerated using a Neobar hemocytometer.

BrdU assay: BrdU cell proliferation assay was done using the BrdU cell proliferation ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 100 μ l of control and treated cells were transferred to 96 wells micro titer plate and 10 μ l of BrdU solution was added to each wells and incubated overnight. Then the microwells were centrifuged and dried by hair drier. Then 200 μ l of FixDenat was added to each well and plate was incubated for 30 min at 15-25°C. After removing FixDenat, 100 μ l/well anti-BrdU-Peroxidase conjugated antibodies were added and incubated 90 min at room temperature. The solution was completely removed and the wells were washed three times with 200 μ l of washing buffer. Finally, 100 μ l of Tetra-Methyl Benzidin (TMB) substrate solution was added and incubated at room temperature for 15 min and reaction was stopped by adding 100 μ l of stop solution. The absorbance was measured using microtiter plate reader at 450 nm Titretek multiscan ELISA reader (Labsystems Multiskan, Roden, Netherlands).

MTT assay: After 120 hr of incubation, 100 μ l of finely resuspended control and Nitric Oxide treated cells were transferred to flat bottom 96 microtiter plates. Then 10 μ l of freshly prepared (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, U.S.A.) solution (5 mg/ml in PBS) was added to each well and were incubated for 4hr. Finally, 50 μ l of MTT lysis solution (20% Sodium Dodecyl Sulphate W/V and 50% Dimethyl Formamide V/V) was added to each well and incubated overnight. Absorbance was read at 620 nm using a Titretek multiscan ELISA reader (Labsystems Multiskan, Roden, Netherlands).

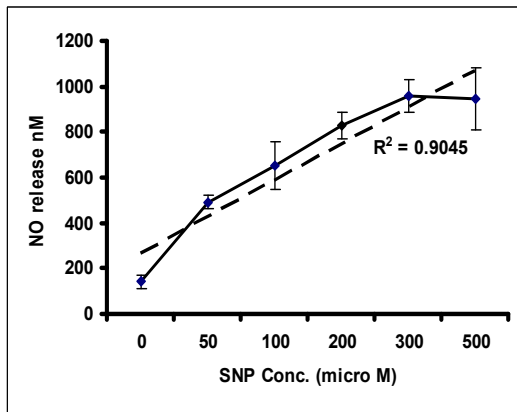
Senescence sensitive β -Galactosidase staining

Cytochemical staining for acidic senescence associated β -Galactosidase (SA- β -gal) was performed as described by Gary and Kindell (2005) with some modification.(28) In a brief HSCs were treated by NO donors and incubated 120 hr. Then the cells were collected and transferred to glass slides by centrifugation with Cytospin (Shandon, Sewickley, PA, USA). After that the cells were fixed for 3-5 min in 2% Formaldehyde, 0.2 % Glutaraldehyde in phosphate buffer saline (PBS) and incubated for 12 hr at 37°C with fresh β -Galactosidase staining solution: 1 mg of 5-Bromo-4-Chloro-3-Indolyl- β -D- Galactopyranose (Sigma, St. Louis, MO, U.S.A.), 5mM Potassium

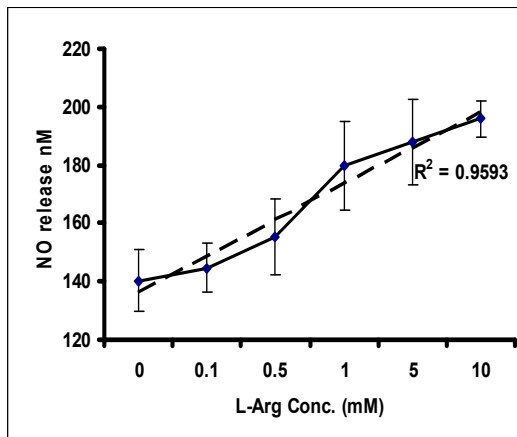
Ferrocyanade, 5mM Potassium Ferricyanade, 2mM MgCl₂, pH 6.0. Finally, the absolute of blue stained cells were counted out of 300 cells.

Telomerase assay

A highly sensitive photometric enzyme immunoassay was used for the detection of telomerase activity. The method is on the base of Telomeric Repeat Amplification Protocol-Polymerase Chain Reaction (TRAP-PCR) that described by Keith and Monaghan (2004).(29) For assay we used TRAP-PCR-ELISA kit (Roche, Mannheim, Germany). The assay separated in two steps. In the first step, telomerase adds telomeric repeats (TTAGGG) to the biotin labeled synthetic P1-TS-primer and then, the elongation product are amplified using the primers P1-TS. In the second step PCR product is denatured and hybridized to a digoxigenin-(DIG)- labeled, telomeric repeat-specific detection probe. The resulting product is immobilized via the biotin labeled primer to a coated microplate. The immobilized PCR product is then detected with an antibody against DIG-POD that is conjugated to peroxidase. Finally, the probe is visualized by virtue of peroxidase metabolizing TMB. Briefly, 2 \times 10⁴ cells were washed with cold PBS and lysed in 200 μ l of pre-cooled lysis solution and incubated on ice for 30 min. Then the lysate was centrifuged at 16,000 x g for 20 min at 4°C. For PCR amplification, 2 μ l of supernatant and 25 μ l of reaction mixture (containing telomerase substrate, primers, nucleotides, and Taq polymerase) were transferred into a suitable tube, and then sterile water was added to a final volume of 50 μ l. An amplification reaction was carried out by PCR. The 5 μ l amplification product was mixed with 20 μ l of denaturation reagent and incubated at room temperature for 10 min and 225 μ l of hybridization buffer was added and mixed thoroughly, then 100 μ l of mixture per well was transferred into the precoated MTP modules and incubated at 37°C on a shaker for 2 h. Anti-DIG-POD working solution (100 μ l) was added and incubated at room temperature for 30 min with shaking. The solution was removed completely and the precipitate was rinsed five times with 250 μ l of washing buffer per well for a minimum of 30 sec. After removing the washing buffer, 100 μ l of TMB substrate solution was added and incubated for color development at room temperature for 20 min with gentle shaking. Finally, 100 μ l of stop reagent was added to each well to stop color development. The amount of TRAP products was determined by the measurement of the absorbance at 450 and



(a)



(b)

Figure- 1: Measurement of NO production by by Greiss reaction. after treatment of HSCs by L-Arg or SNP. Cord blood HSCs were treated by 0-10 mM of L-Arg or 0-500 μ M of SNP in RPMI-1640 medium. Cell-free supernatants were recovered after incubation. Produced NO was quantitatively measured as NO_3^- (Nitrate: a stable metabolite of NO) and NO_2^- (Nitrite) as described in methods. **a)** NO production by HSC in the presence of SNP. **b)** NO production by HSC in the presence of L-Arg. Each point represents the means of quadruplicate determinants. The standard deviations were less than 8% of the means in each case.

690nm by using Titertek multiscan ELISA reader (Labsystems Multiskan, Roden, Netherlands). A negative as well as a positive control was run each time. A negative control was provided for each extract by heat inactivating the telomerase enzyme present in cell lysate at 65°C for 10 min prior to the PCR step.

Semi-quantitative detection of Nitric Oxide Synthase (iNOS) mRNA by RT-PCR

Nitric Oxide treated HSCs cells were collected and total RNA was extracted by using the Tripur isolation reagent (Roche, Mannheim, Germany), according to the manufacture's protocol. RNA yield and purity were quantitated by measuring optical density ($\text{OD}_{260/280}$) using a spectrophotometer Beckman DU530 (Beckman Coulter Inc. CA, USA). First-strand cDNA was synthesized from 1

μ g of total RNA using Maurine Maloney Leukemia virus (M-MLV, Fermentas) and reverse transcriptase (Fermentas GmbH, Leon-Rot, Germany) with oligo-dT primer (Fermentas GmbH, Leon-Rot, Germany), according to the manufacture's instructions.

The iNOS cDNA and β -actin cDNA were amplified by the following primers; iNOS forward PCR primer:

5'-
CGGTGCTGTATTTTCCTTACGAGGCGAAGAA
GG-3', reverse primer

5'-
GGTGCTACTTGTTAGGAGGTCAAGTAAAGG
GC-3'; β -actin forward 5'-
AAGAGAGGCATCCTCACCCCT-3' and reverse 5'-
TACATGGCTGGGGTGTGAA-3'.

The expected sizes of the PCR product were 257 bp for iNOS and 218 bp for β -actin. The thermal cycling conditions for amplification of the iNOS (257-bp) and β -actin (218-bp) fragments were as follows: 94°C for 10 min., followed by 35 cycles at 94°C for 50s; 54°C for 30s; 72°C for 60s. This was followed by re-extension at 72°C for 10 min.

The PCR products were separated on a 2% agarose gel (using 0.5 \times TBE buffer) and visualized by ethidium bromide staining.

Statistical analysis

Each experiment was minimally performed three times for all data, each carried out in duplicated sequences. Data were analyzed using a One-Way Analysis of variance (ANOVA) Values were given as the mean \pm Standard Deviation (SD) and analytical variables were compared by using the students' t-Test. By convention, a α -level of $p < 0.05$ was considered to be statistically significant.

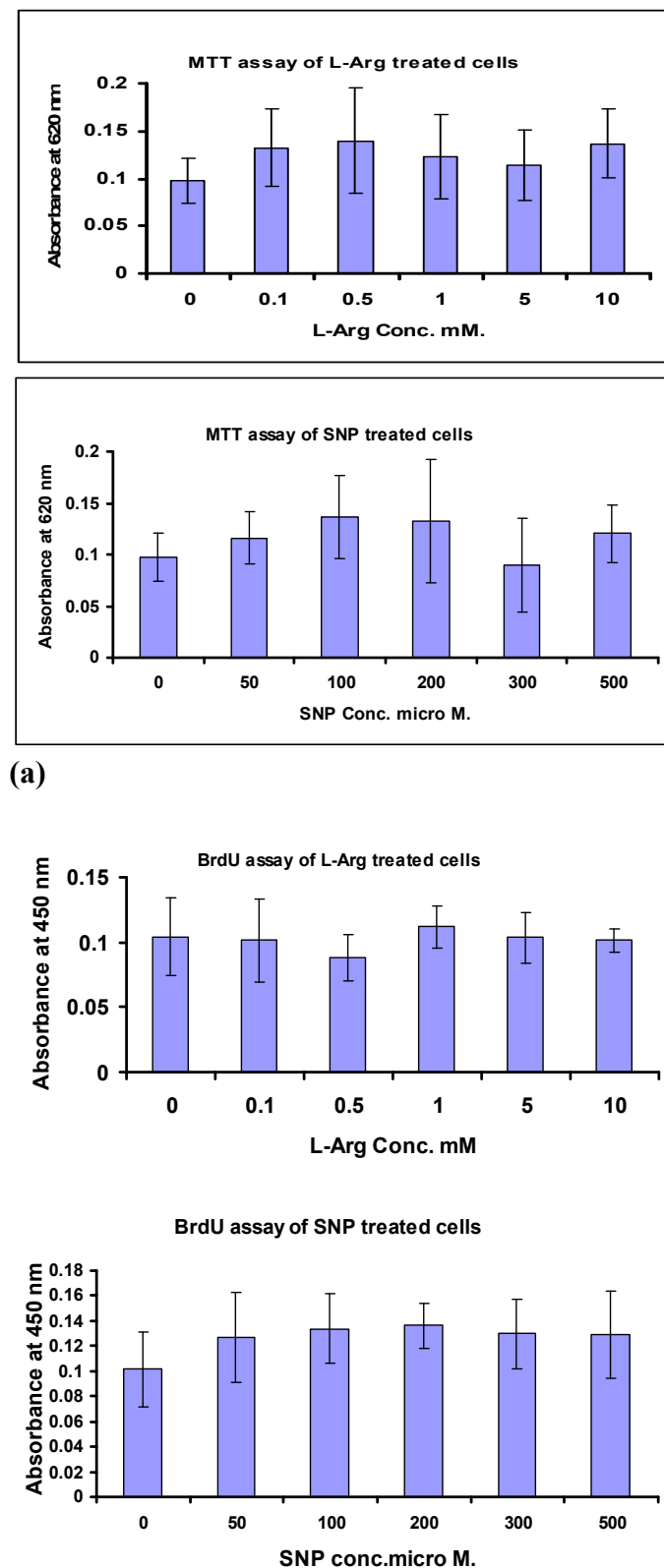
Results

NO production by HSCs

In this work, the response of Hematopoietic stem cells to L-Arginine and sodium Nitroproside (as NO donors) was studied.

The cells' supernatants were collected after treatment by L-Arg and SNP after 120 hr. The supernatants were assessed for yield of released NO.

The assays were carried out as explained in methods those factors should tightly controlled to prevent failure of the assay. An assay failure was defined as the inability to generate a standard curve in the range of 0.05-2.0 AU.



(b) **Figure- 2:** Effect of Nitric Oxide on the cell growth and cell proliferation of the HSCs. HSCs were treated by 0-10 mM of L-Arg or 0-500 μ M of SNP in RPMI-1640 medium for 120 hr. Cell proliferation was assayed by MTT and BrdU assays as described in methods. **a)** MTT assays of HSC after treatment by L-Arg or SNP. **b)** BrdU assays of HSC after treatment by L-Arg or SNP. Each point represents the means of quadruplicate determinants.

Initially the standard calibration curves of Sodium Nitrite and Sodium Nitrate were plotted. The

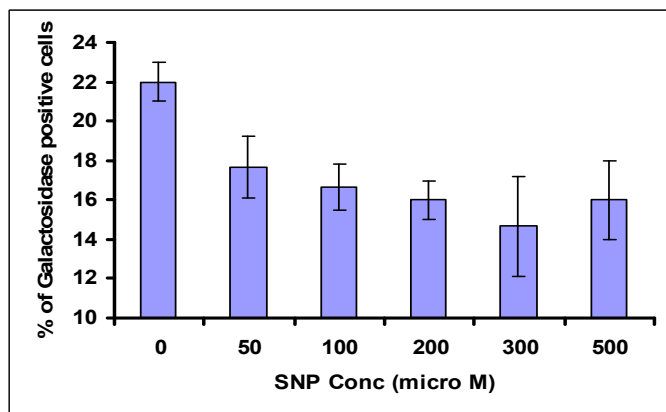
measured absorbance was plotted against the sodium Nitrite or Sodium Nitrate concentrations. A higher degree of correlation was observed in standard curves (Data was not shown). When the HSCs were treated by SNP or L-Arg, the amount of released NO in culture medium was quantified by measurement of NO metabolites (Nitrate and Nitrite) as described in methods. HSCs those treated with SNP exhibited an increase 3-7 fold (400-1000 nM) in NO production in comparison to untreated control cells (140 nM) Figure 1a. Treatment of cells by L-Arg resulted lower release of NO (Up to 200 nM) in comparison to SNP. The results indicated that released NO was increased about 2 fold with increasing L-Arg concentration as compared to that of the control cells and this increase was dependent to the L-Arg concentration too (Figure 1b).

Cellular viability and cell proliferation after treatment by NO

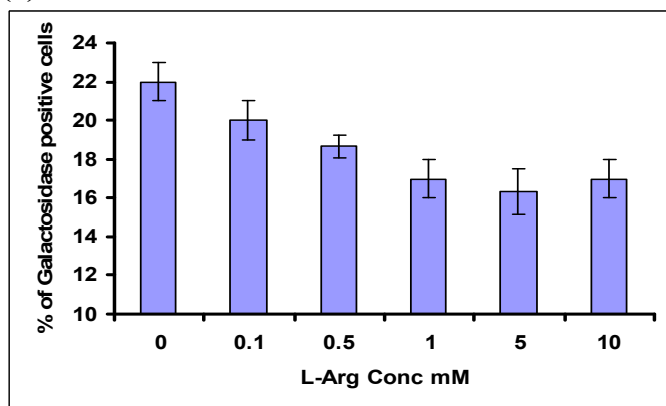
To assess the effects of SNP and L-Arg (as NO donors) on hematopoietic stem cell growth and viability, initially we determined the probable cytotoxic effects of these compounds on the cells by using trypan blue exclusion test, cell counting, MTT and BrdU cell proliferation assays. When the viability was assayed after 120 hr incubation, the results indicated that cells were still viable more than 90% after treatment with SNP (up to 500 μ M) or L-Arg (up to 10 mM) (Data was not shown). This indicated that in these conditions SNP and L-Arg have not cytotoxic effects on HSCs. The cell proliferation and growth rate of SNP and L-Arg treated HSCs were assessed using two colorimetric (BrdU and MTT) cell proliferation assays and cell counting at each concentration after 120 hr. The values for different cells were averaged and growth curves were constructed. The results of MTT (Figure 2a) and BrdU cell proliferation assays (Figure 2b) were shown in figure 2. The results indicated to the increasing of cell growth potential and cell proliferation parameters up to 40% in the presence of NO donors in comparison of untreated control cells.

Senescence sensitive β -Galactosidase staining

To examine the effects of NO on the onset of HSCs senescence, cells were incubated with the NO donors for 120 hr. Light microscopy confirmed the slightly inhibition of senescence with typical features of senescence including an increased cell size and cytoplasmic granularity in senescence cells (Data not shown). Figure 3a illustrate that the addition of SNP reduced the number of senescence



(a)



(b)

Figure- 3: Effect of NO on senescence of HSCs. HSCs were treated by NO donors and incubated 120 hr. Then the cells were collected and transferred to glass slides by centrifugation with Cytospin. After that the cells were fixed and incubated for 12 hr at 37°C with fresh β -Galactosidase staining solution. Finally, the absolute of blue stained cells were counted out of 300 cells. **a)** Percent of senescent HSCs in the presence of SNP. **b)** Percent of senescent HSCs in the presence of L-Arg. Each point represents the means of triplicate determinants.

β -Galactosidase positive cells from 22% \pm 1.0% in control cells to 15 \pm 1.5% with maximal effect at 300 μ l of SNP. L-Arginine reduced number of β -Galactosidase positive cells from 22% \pm 1.0% in control cells to 17 \pm 1.5% with maximal effect at 5mM of L-Arg concentration Figure 3b.

Effect of NO on the telomerase activity

To examine the effect of NO on the telomerase activity, the activity of enzyme was assayed by TRAP-PCR ELISA method as described above. The telomerase activity was analyzed over 120 hr incubation of cells after treatment by SNP and L-Arg. Treatment of cells by NO donors slightly induced the telomerase activity of the cells. When the telomerase activity was measured after treatment, the activity of enzyme was increased up to 25% in the presence of 100 μ M of SNP or 1.0 mM of L-Arg in comparison to control cells (Figure 4). These results suggest that SNP and L-Arg were probably induced HSCs I growth through inducing

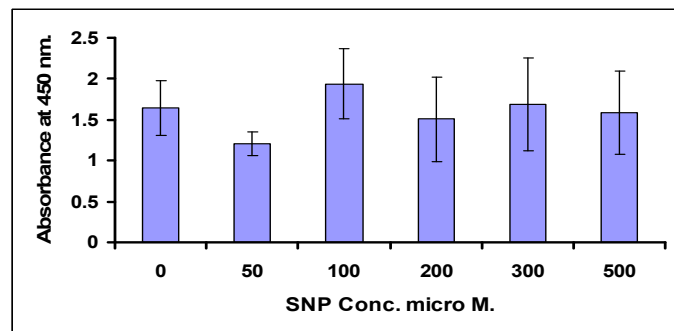
telomerase activity followed by treatment by NO donors. Importantly, slight inhibition of HSCs senescence was accompanied by a significant induction of the telomerase activity.

Effect of *L-arg* and *SNP* on *iNOS* mRNA Expression

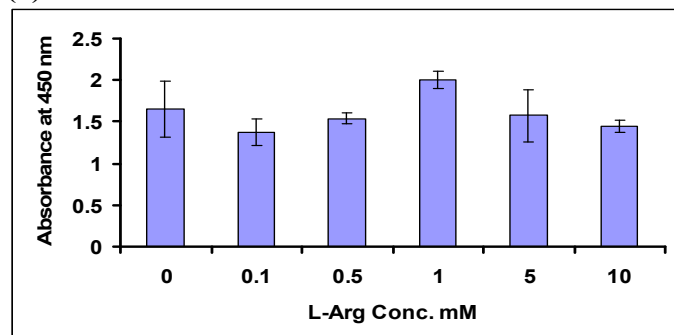
So as to examine level of iNOS mRNA expression in HSCs in response to SNP or L-Arg real-time PCR was performed using RNA from untreated and treated cells. The quantity of the target was normalized by an endogenous reference (β -actin) relative to the calibrator (untreated cells). This method revealed the expression of iNOS mRNA, as defined by a 257 bp PCR product in the HSCs. Control cells (Untreated cells) showed weak expression of iNOS Figure 5. The results presented in figure 5a show that L-Arginine treatment increased iNOS mRNA by 2-10 fold compared with control cells. The highest iNOS mRNA expression was observed in 5 and 10 mM of L-Arg. These results indicated that the profile of NO production is similar to the iNOS expression. Moreover, is dependent to the iNOS expression and iNOS activity. Treatment of HSCs by SNP did not increase the iNOS expression significantly. The iNOS mRNA relative level was not increased in comparison with untreated and house keeping gene (β -actin) mRNA level Figure 5b.

Discussion

This study was focused on attempting to understand the effect of the Nitric Oxide on the cell proliferation and telomerase activity of human cord blood hematopoietic stem cells. We have treated the CB HSCs with increasing concentrations of L-Arginine and Sodium Nitroproside up to 120 hr. The present work demonstrates that NO affect telomerase activity and cellular replicative capacity in human hematopoietic stem cells. A significant behavior was observed on the telomerase activity and cell proliferation after treatment of cells This conclusion is based on the following findings: first, NO can increase telomerase activity up to 25% in comparison to untreated control cells. Second, significant induction of the telomerase activity was accompanied by a slight inhibition of HSCs senescence. Third, the telomerase induction and reduce cell senescence were accompanied by increasing cell proliferation parameters. Finally, these phenomenons were occurred without significant induction in the nitric oxide synthetase expression.



(a)



(b)

Figure- 4: Effect of Nitric Oxide on the telomerase activity of the HSCs. HSCs were treated by NO donors (L-Arg or SNP) and incubated 120 hr. Then the cells were collected and total cell protein was extracted and telomerase activity was assayed by Telomeric Repeat Amplification Protocol- Polymerase Chain Reaction ((TRAP-PCR) ELIZA method as described in methods. **a)** Telomerase activity of HSC after treatment by SNP for 120 hr. **b)** Telomerase activity of of HSC after treatment by L-Arg for 120 hr. Each point represents the means of quadruplicate determinants.

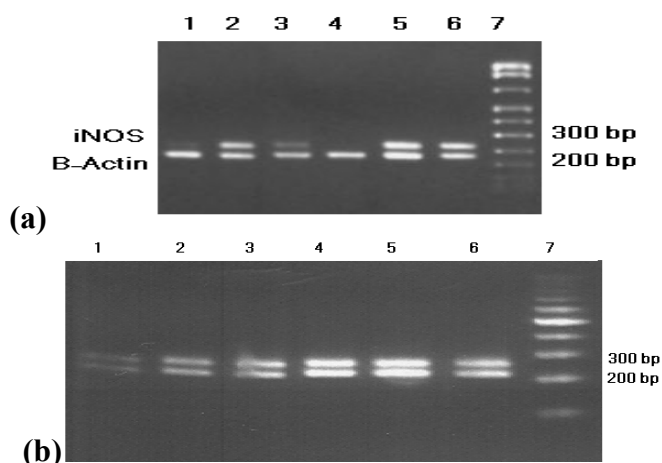


Figure- 5. Effect of L-Arg or SNP on the expression of iNOS. Nitric Oxide treated HSCs cells were collected and total RNA was extracted. RNA yield and purity were quantitated by measuring optical density ($OD_{260/280}$) using a spectrophotometer. First-strand cDNA was synthesized from 1 μ g of total RNA and reverse transcriptase with oligo-dT primer as described in methods. The iNOS cDNA and β -actin cDNA were amplified by the specific primers. The expected sizes of the PCR product were 257 bp for iNOS and 218 bp for β -actin. The PCR products were separated on a 2% agarose gel (using 0.5 \times TBE buffer) and visualized by ethidium bromide staining. **a)** RT-PCR of iNOS from HSCs after treatment by SNP for 120 hr. **b)** RT-PCR of iNOS from HSCs after treatment by L-Arg for 120 hr.

The effect of NO on telomerase activity has examined in different cells. In rat neural precursor cells, low concentrations of NO donor (DETA-NO) were reported to cause a decrease in telomerase activity. This phenomenon was occurred concomitantly with the NO-induced inhibition of cell proliferation and the promotion of neuronal differentiation.(30) Thus, this inhibitory action of NO on telomerase does not conflict with our findings, but is consistent with the notion that the enzyme is down-regulated when cells withdraw from the cell cycle.(31) In apparent discrepancy with our results, a study on the effect of NO has previously suggested that NO activates telomerase and delays replicative senescence of human umbilical vein endothelial cells (HUVEC).(32) But in another work, NO does not affect telomerase activity and cellular replicative capacity of HUVEC cells.(33) The reasons for the discrepancy between the results of HUVEC and HSCs studies are presently unclear. Particularly since in both instances the concentrations, cell type and time of exposure at which pharmacological agents were tested were comparable. It could be argued that in studies reporting negative results subtle experimental differences might have affected NO production and/or its bioavailability, thus rendering the treatments ineffective. In this respect, it should be noted that our studies were initiated with HSCs of a low replicative age and were expanded in the presence of EPO, IL-3, IL-6 and GM-CSF.

Senescence is believed to be triggered by a critical decrease in telomerase activity and shortening of telomere lengths. Most cycling HSCs display telomerase activity. Following an initial increase, telomerase activity is down regulated as HSCs proliferate and differentiate into more mature cells that display low to negligible levels of telomerase activity.(34) Telomerase expression and telomerase activity in the human HSCs constitutive possibly due to dysregulation of a telomerase activity factor such as c-myc, since over expression of c-myc in normal hematopoietic cells leads to telomerase activation and cellular immortalization.(35) HSC self-renewal is not a perfect process and daughter cells have progressively reduced proliferative capacity, due in part to progressive telomere erosion with each cell division. This, in turn, leads to proliferative senescence that can be observed both *in vivo* and *in vitro*.(36)

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