Research Article

Effect of Retinoic Acid in Improving Endothelium Dependent Vascular Reactivity in Hyperhomocysteinemic Rats

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Abstract

Introduction: Retinoic acid is a metabolite of Vitamin A (retinol) has been reported to activate PI3K/Akt pathway and up regulate NO synthesis that has a beneficial effect in the endothelial cell. Thus, the present study was designed to investigate the effect of retinoic acid in HHcy induced vascular dysfunction.

Objective: This study evaluates the protective effect of retinoic acid on L-methionine-induced Hyperhomocysteinemia (HHcy) in rats.

Materials and methods: Wistar rats were randomly divided into seven groups as normal control, L-methionine, HHcy+Retinoic acid (2.5 mg/kg/d), HHcy+RA (5 mg/kg/d), HHcy+RA (5 mg/kg/d) + BEZ235 (4 mg/kg/d), HHcy+RA (5 mg/kg/d)+L-NAME (10 mg/kg/d), HHcy+Lisinopril (1 mg/kg./d). HHcy was induced by oral administration of L-methionine (1.7 g/kg) for 28 days. From the 21st day of study retinoic acid (2, 2.5 mg/kg), BEZ235, L-NAME, Lisinopril was administered to L-methionine-treated rats. Different biomarkers of HHcy in serumand vascular reactivity were evaluated.

Results: L-methionine administration, vascular endothelium dysfunction was assessed in terms of attenuation of acetylcholine-inducedendothelium-dependent relaxation (Isolated aortic ring preparation), a decrease in serum nitrite level, mRNA expression of eNOS (rtPCR). Administration of retinoic acid (2.5 mg/kg/d and 5 mg/kg/d, 21st to 28th day) significantly improved acetylcholine-induced endothelium-dependent relaxation, serum nitrate/nitrite level, mRNA expression of eNOS. This ameliorative effect of retinoic acid was blocked by BEZ235 (a PI3K /mTOR dual inhibitor) and L-NAME (NOS inhibitor).

Conclusion: Retinoic acid attenuates hyperhomocysteinemia induced vascular endothelium dysfunction in Wistar rats by activation of PI3K/mTOR and eNOS pathway.

Keywords: Retinoic acid; Endothelium dysfunction; Hyperhomocysteinemia; L-Methionine

Introduction

In cardiovascular system, vascular endothelium performs various important functions. It includes new blood vessels formation, control capillary permeability, inflammation, coagulation, maintains vascular tone in normal as well as diseased states. Vascular endothelium dysfunction is characterized by a shift of endothelial actions toward reduced vasodilatation, prothrombotic and pro-inflammatory properties. It has been documented that Hyperhomocysteinemia (HHcy) leads to the impairment of vascular function and is a putative risk factor for cardiovascular and cerebrovascular disorders [1].

Homocysteine (Hcy), a sulfur-containing amino acid which is formed during methionine metabolism. Homocysteine metabolism occurs by remethylation pathway and transculturation pathway. Cofactors like cobalamine (vitamin B12), folate, or pyridoxine (Vitamin B6) are also involved in Hcy metabolism. An alteration in these enzymes and nutritional deficiency of essential cofactors or enzyme substrates leads to the elevation in plasma homocysteine level as a result of attenuation of homocysteine metabolic pathways that leads to the Hyperhomocysteinemia (HHcy) [2]. Elevated plasma homocysteine level is contributed in plaque and it has been documented that it to attenuate endothelium-dependent, flow-mediated dilatation [3]. Elevated homocysteine levels lead to the generation of reactive oxygen species viz. superoxide anions and hydrogen peroxide that causes nitric oxide degradation [4]. HHcy is associated with activation of Asymmetric Dimethyl Arginine (ADMA) that is competitive inhibitor of eNOS and leads to the deficiency of Nitric Oxide (NO) that contributes to vascular endothelium dysfunction.

At the molecular level HHcy cause modulation of eNOS by an imbalance of phosphorylation and dephosphorylation status of lipid and protein kinase that contributes to vascular endothelium dysfunction. Retinoic acid, a metabolite of vitamin A that are mainly involved in regulation of cell development, cell differentiation, proliferation, embryogenesis,

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hematopoiesis, reproduction, vision, maintenance of immune function, spermatogenesis and apoptosis. Synthesis of Retinoic acid occurs locally and released in an autocrine and paracrine fashion just like hormones from liver and kidney. Retinoic acid functions are mediated by nuclear receptors, specifically by retinoic acid receptors (RAR α, β, and γ) and the retinoic X receptors (RXR α, β, and γ). RARs act as retinoid inducible transcriptional factors and can form heterodimers with RXRs, which regulate the expression of genes involved in cell cycle arrest, cell differentiation and cell death [5]. RA increases NO production by eNOS phosphorylation through RAR-mediate deactivation of the PI3K/Akt pathway in vascular ECs and possibly plays a beneficial role in the vascular endothelium. Retinoic acid has been reported to activate PI3K/Akt pathway and up regulate NO synthesis and has a beneficial effect in endothelial cell. [6] Retinoic acid has also been reported to decrease the Hcy level [7]. Retinoic acid has been documented to stimulate mTOR and inhibit the apoptosis and NF-kB and shows cardiovascular protection. Therefore, the present study is designed to investigate the effect of Retinoic acid in HHcy induced vascular endothelium dysfunction.

Materials and Methods

Animals

The study was carried out in, age-matched, Wistar rats, weighing 180 g-220 g, The rats were maintained at standard conditions of temperature (25ºC) and humidity (45%-55%) and housed under the 12 h light, 12 h dark cycle. The rats were fed on standard chow diet (Ashirwad Industries, Ropar, India) and Water ad libitum. Body weights and food intake were measured daily during the study. The experimental protocol was approved by Institutional Animal Ethical Committee in accordance with guidelines of Indian National Science Academy (INSA).

Drugs and chemicals

Retinoic acid and L-Methionine were purchased from Sigma-Aldrich (Bangalore, India).

Induction of experimental hyperhomocysteinemia

Rats were fed with L-Methionine diet (1.7 g/kg/day, per oral) for 4 weeks to induce hyperhomocysteinemia and endothelial dysfunction (Figure 1) [8].

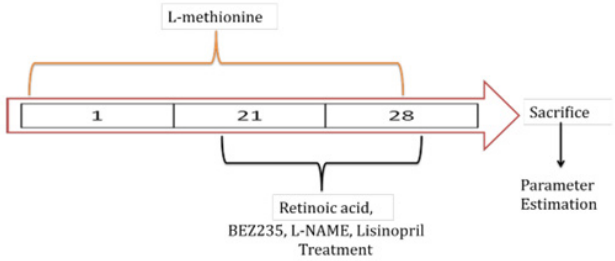

Figure 1. Treatment schedule of drugs.

Experimental design

Wistar rats were divided into seven groups, and each group consists of 6 animals (n=6). Retinoic acid and L-methionine were freshly prepared before administration. L-methionine, retinoic acid, BEZ235, lisinopril were administered by oral route and L-NAME was administrated by i.p. route.

Group I: Normal control.

Group II: L-Methionine (1.7 g/kg/day) was administered for 4 weeks to induce HHcy.

Group III: Retinoic acid was administered at 2.5 mg/kg/day from the 21st day of L-methionine treatment for one week.

Group IV: Retinoic Acid (RA) was administered at 5 mg/kg/day from the 21st day of L-methionine treatment for one week.

Group V: Retinoic acid and BEZ235 was administered at 5 mg/kg/day and 4 mg/kg/day respectively, from the 21st day of L-methionine treatment for one week.

Group VI: Retinoic acid and L-NAME was administered at 5 mg/kg/day and 10 mg/kg respectively, from the 21st day of L-methionine treatment for one week.

Group VII: Lisinopril 1 mg/kg/day were administered from the 14th day of L-methionine treatment for one week.

Biochemical analysis

At the end of the experiment (28th day), blood sample from retro-orbital puncture was collected in clean dry centrifuge tubes for biochemical estimation. The blood was centrifuged at 4000 rpm for 15 min, at room temperature for the separation of a clear, non-hemolyzed supernatant serum, aliquoted and stored at -20ºC until analysis was carried out.

Later, all rats were sacrificed by cervical dislocation and blood vessels (n=6) were isolated for biochemical analysis and assessment of vascular reactivity.

Estimation of serum homocysteine

Derivatization method: The following fluids were sequentially combined: 0.5 ml 0.5 M Tris-HCl buffer, pH 8.9; 130 µl acid supernatant fluid, 20 ml of 400 µmol/ l D-penicillamine and 350 µl of 10 mM DTNB in 0.5 MK_2HPO_4, pH 7.2. After 5 min, the mixture was 24 re-acidified by the addition of 50 ml of 7 MHPO, centrifuged and analyzed by HPLC. The re-acidified derivatives were stable for at least 36 h at room temperature. The reaction mixture for the analysis of total sulf-hydryls consisted of 0.5 ml 0.5 M Tris-HC1buffer, pH 8.9; 150 µl of acid supernatant fluid, 20 µl of 400 µmol/ l D-penicillamine and 20 µl of 10 mM dithiothreitol. After 5 min, 350 µl of 10 mMDTNB in 0.5 M K_2HPO_4, pH 7.2 was added. After an additional 5 min, the reaction mixture was re-acidified by the addition of 50 µl of 7 M H_3PO_4, centrifuged and analyzed by HPLC.

Chromatography method

Chromatography of the sulfhydryl-DTNB derivatives was accomplished using isocratic elution on anLC-18T column (150 × 34.6 mm, 3 µm) at 37ºC. Mobile phase A consisted of 12% methanol (v/v), 100 m MKH_2PO_4, pH 3.8 at a flow rate of 1.2 ml/min. Mobile phase B consisted of 40% methanol (v/v), 100 m MKH_2PO_4, pH 3.8 at a flow rate of 1.2 ml/min. Analysis was initiated by the injection of 20 µl of the re-acidified derivatization reaction. Sulphydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nm. After 10 min of isocratic elution with mobile phase A the eluent was changed

to mobile phase B in a linear manner during 1 min using the system controller. Mobile phase B was used for 8 min to elute excess DTNB reagent from the column and then the column was re-equilibrated for 7 min with mobile phase A before injection of the next sample [9].

Assessment of vascular endothelial dysfunction

Ach-induced endothelium-dependent and SNP-induced endothelium-independent relaxation on isolated rat aorta preparation.

The rats were sacrificed by cervical dislocation, followed by decapitation. The aorta was exposed and carefully dissected out. The aorta was placed in ice cold aerated Krebs-Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 1.0 mM; glucose, 11.1 mM; KH_2PO_4, 1.2 mM and $CaCl_2$, 2.5 mM) and connective tissue was removed. Isolated aorta was cut into 1.5-2 mm wide ring and one ring was mounted in myograph containing Krebs-Henseleit solution maintained at 37°C, pH 7.4 and bubbled with the carbogen (95% O_2 and 5% CO_2). The isometric contractions were recorded with a force transducer connected tomograph. The aortic ring preparation was primed with 80 m MKCl to check its functional integrity and to improve its contractility. The cumulative dose response of Ach (10^{-8} M to 10^{-4} M) or Sodium Nitroprusside (SNP) (10^{-8} M to 10^{-4} M) wer e recorded in phenylephrine (3×10^{-6} M) pre-contracted preparation with intact or denuded endothelium, respectively [10].

Estimation of serum nitrite concentration

Serum nitrite concentration was estimated using Greiss reagent. 400 µl of carbonate buffer (pH 9.0) was added to 100 µl of serum or standards sample followed by addition of small amount (~0.15 g) of copper-cadmium alloy. The tubes were incubated at room temperature for 1 h to reduce nitrate to nitrite. The reaction was stopped by adding 100 µl of 0.35 M sodium hydroxide. Following this, 400 µl of zinc sulfate solution (120 mM) was added to deproteinate the serum samples. The samples were allowed to stand for 10 minutes and then centrifuged at 4000 g for 10 min. Greiss reagent (250 µl of 1.0% sulfanilamide prepared in 3 N HCl and 250 µl of 0.1% N-naphthyl ethylene diamine prepared in water) was added to aliquots (500 µl) of clear supernatant and serum nitrite was measured spectrophotometrically at 545 nm. Standard curve of sodium nitrite (5 µM to 50 µM) was plotted to calculate the concentration of serum nitrite [11].

Assessment of oxidative stress

Estimation of serum Thiobarbituric Acid Reactive Species (TBARS) level: A total of 1 ml of 20% trichloroacetic acid was added to 100 µl of serum and 1% Thiobarbituric Acid (TBA) reagent (1.0 ml), which were mixed and incubated at 100ºC for 30 min. following which the samples were placed on ice and brought down to normal temperature. Samples were then centrifuged at 1000 g for 20 min. Serum concentration of TBARS measured spectrophotometrically at 532 nm. A standard curve using 1, 1, 3, 3-tertramethoxyopropane (1 µM-10 µM) was plotted to calculate the concentration of TBARS [12].

Reduced Glutathione (GSH): To measure the reduced glutathione level, Aortic tissue homogenate (in 0.1 M phosphate buffer ph 7.4) was taken. The procedure was followed initially as described by Ellman, 1959. The homogenate was added with equal volume of 20% Trichloroacetic Acid (TCA) containing 1 mm EDTA to precipitate the tissue proteins. The mixture was followed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant (200 µl) was then transferred to a new set of test tube and added 1.8 ml of the Ellman reagent (5,5-dithiol bis-2-nitrobenzoic acid) (0.1 mm) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution. Then all the test tube makes up the volume of 2 ml. After the completion of the total reaction, solution was measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH. The level of GSH was expressed as µmol/mg of Aortic wt [13].

Molecular assays

Assessment of mRNA expression of eNOS: 100 mg of aortic tissue was homogenized in 1 ml of triazole reagent and incubated for 5 min at 20ºC. 0.2 ml of chloroform was added to homogenate, incubated at 20ºC for 3 min. mixture was centrifuged at 10,000 g for 15 min at 4ºC ,upper aqueous phase was isolated and 0.5 ml isopropyl alcohol was added to precipitate RNA. The sample was centrifuged at 10,000 g for 15 min at 4ºC to form gel-like pellet of RNA in the tube. The supernatant was removed, RNA pellet was washed with 75% ethanol, mixed, centrifuged at 7,500 g for 5 min at 4ºC and RNA pellet were briefly vacuum dried for 5 min-10 min. The RNA was quantified by ultraviolet absorbance spectrophotometry toascertain A260/A280 ratio<1.6 and dissolved in RNase-free water. 5 µl reverse primer was added to crude RNA, 29 µl reverse transcriptase buffer incubated for 10 min at 65ºC and cooled on ice. 16 U AMV transcriptase (10 U/µl) and 5 µl 10 nM dNTP mixture were added, incubated at 42ºC for 1 hr and 10 mM Tris buffer (pH-7.5) was added to synthesize single stranded cDNA. 5µl cDNA product was mixed with 4 µl dNTP mixture, 5 µl forward primer, backward primer, 10 X amplification buffer, 0.9 µl of Taq DNA polymerase enzyme (3U/µl) and 70.1 µl RNase free water in PCR tube and overlaid with 100 µl mineral oil. 24 PCR cycles of GAPDH (94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min) and 30 PCR cycles of eNOS (94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min) followed by 1 cycle at 57ºC for 2 min and 72ºC for 7 min were performed using 1 half of the reverse transcription mixture (Biorad , MJ MiniThermal cycler), sense and antisense primers for eNOS: (5'-TCCAGAAACACAGACAGTGCA-3' and 5'-CAGGAAGTAAGTGAGAGC -3'resp.) and for GAPDH (5'-TCCCTCAAGATTGTCAGCAA -3' and 5'-AGATCCACAACGGATACATT -3' resp.) were used. The PCR products so obtained were analyzed on ethidiumbromide-stainedagarose (1.5%) gel on Gel electrophoresis apparatus (Biorad). The eNOS and GADPH products were quantified using image (Gel Doc EZ image, Biorad) and amount of eNOS was normalized with respect to amount of GAPDH product [14].

Estimation of tumour necrosis factor: Alpha (TNF-α) levels TNF-a level was estimated by using rat TNF-a kit (RayBio, Rat TNF-alpha ELISA kitprotocol) which uses a microtitre plate reader read at 450 nm. Concentrations of TNF-α were calculated from plotted standard curve.

Statistical analysis: All values were expressed as mean ±

S.D. Vascular reactivity of isolated aortic ring preparation was statistically analyzed using one-way ANOVA followed by Newman-Keul's test. All biochemical parameters were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. The p-value<0.05 was considered as statistically significant.

Results

Effect of pharmacological interventions on serum homocysteine level

L-Methionine administration in rats, for 4 weeks, significantly ($p<0.05$) increased serumhomocysteine concentration in comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) significantly ($p<0.05$) reduced serum concentration of homocysteine as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) reverses the protective effect of retinoic acid (Figure 2).

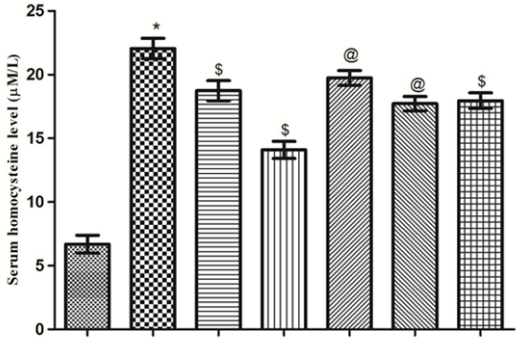

Figure 2. Effect of pharmacological interventions on serum Hcy level. All values (n=6) represent mean ± S.D. p0.05 vs. normal control; $p0.05 vs. HHcy control; @p0.05 vs. RA (5 mg/kg). **Note:** (▩) Normal Control, (▩) HHcy control, (▤) HHcy+RA (2.5 mg/kg), (▥) HHcy+RA (5 mg/kg) HHcy+RA+BEZ235, (▨) HHcy+RA+L-Name, (▦) HHcy+Lisinopril.

Effect of pharmacological interventions on acetylcholine-inducedendothelium-dependent and sodium nitroprusside-induced endothelium-independent relaxation

Acetylcholine and sodium nitroprusside produced endothelium-dependent and independent relaxation respectively, in a dose-dependent manner, in phenylephrine (3×10^{-6}) pre-contracted isolated rat aorta preparation of normal rats. HHcy significantly attenuated acetylcholine-induced endothelium-dependent relaxation. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) significantly ($p<0.05$) improve Acetylcholine-induced endothelium-dependent relaxation in respective groups of hyperhomocysteinemic rats. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) blocked the protective effect of retinoic acid. Lisinopril (1 mg/kg/d, p.o.) treatment also shows the protection. However, no significant effect was observed on SNP-induced endothelium-independent vaso relaxation (Figures 3 and 4).

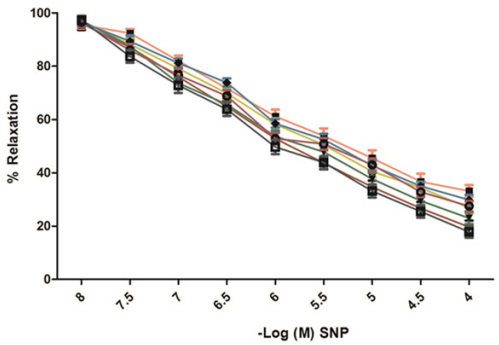

Figure 3. Effect of pharmacological interventions on SNP induced endothelium independent relaxation in phenylephrine precontracted isolated aortic ring preparation. All values (n=6) represent mean ± S.D. **Note:** (●) Normal, (■) HHcy Control, (▲) HHcy+RA (2.5 mg/kg), (▼) HHcy+RA (5 mg/kg), (◆) HHcy+RA+BEZ235, (●) HHcy+RA+L-Name, (▬) HHcy+Lisinopril.

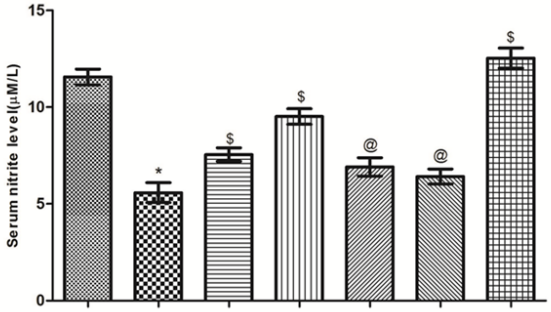

Figure 4. Effect of pharmacological interventions on serum nitrite level. All values (n=6) represent mean ± S.D. p0.05 vs. Normal control; $p0.05 vs. HHcy control; @p0.05 vs. RA (5 mg/kg). **Note:** (▩) Normal Control; (▩) HHcy control (▤) HHcy+RA (2.5 mg/kg); (▥) HHcy+RA (5 mg/kg) HHcy+RA+BEZ235; (▨) HHcy+RA+L-Name; (▦) HHcy+Lisinopril.

Effect of pharmacological interventions on serum nitrite/nitrate concentration

Hyperhomocysteinemia significantly reduces serum nitrite/nitrate concentration in comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) significantly ($p<0.05$) improve serum nitrite concentration as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) attenuate the protective effect of retinoic acid. Lisinopril (1 mg/kg/d, p.o.) treatment also shows the protection (Figure 5).

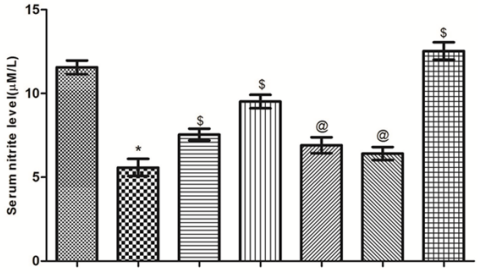

Figure 5. Effect of pharmacological interventions on serum nitrite level. All values (n=6) represent mean ± S.D. p0.05 vs. Normal control; $p0.05 vs. HHcy control; @p0.05 vs. RA (5 mg/kg). **Note:** (▩) Normal Control; (▩) HHcy control; (▤) HHcy+RA (2.5 mg/kg); (▥) HHcy+RA (5 mg/kg) HHcy+RA+BEZ235; (▨) HHcy+RA+L-Name; (▦) HHcy+Lisinopril.

Effect of pharmacological interventions on serum TBARS level

Administration of L-Methionine in rats, for 4 weeks, significantly increased serum TBARS concentration in comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) significantly ($p<0.05$) reduced serum TBARS level as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) prevents the beneficial effect of retinoic acid. Lisinopril (1 mg/kg/d, p.o.) treatment also shows the protection (Figure 6).

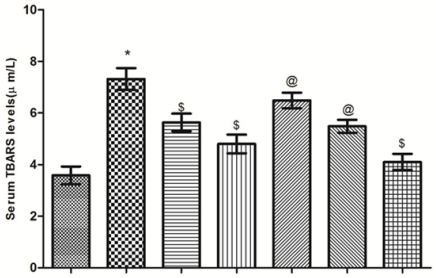

Figure 6. Effect of pharmacological interventions on serum TBARS levels. All values (n=6) represent mean ± S.D. p0.05 vs. Normal control; $p0.05 vs. HHcy control; @p0.05 vs. RA (5 mg/kg). **Note:** ( ) Normal Control; ( ) HHcy control; ( ) HHcy+RA (2.5 mg/kg); ( ) HHcy+RA (5 mg/kg) HHcy+RA+BEZ235; ( ) HHcy+RA+L-Name; ( ) HHcy+Lisinopril.

Effect of pharmacological interventions on reduced glutathione level

Administration of L-Methionine in rats, for 4 weeks, significantly decreased reduced glutathione level in comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) and Lisinopril (1 mg/kg/d, p.o.) significantly ($p<0.05$) increase reduced glutathione level as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) attenuate the protective effect of retinoic acid.

Assessment of mRNA expression of eNOS

Administration of L-Methionine in rats, for 4 weeks, significantly decreased the expression ratio of eNOS/GAPDH in comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) and Lisinopril (1 mg/kg/d, p.o.) significantly ($p<0.05$) improve the mRNA expression of eNOS as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) attenuate the protective effect of retinoic acid.

Estimation of tumour necrosis factor-alpha (TNF-α) levels

Administration of L-Methionine in rats, for 4 weeks, significantly increased the TNF-α expression in hyper homocysteinemic rats as comparison to normal control. Administration of retinoic acid (2.5 mg/kg/d, p.o. and 5 mg/kg/d, p.o.) and Lisinopril (1 mg/kg/d, p.o.) significantly ($p<0.05$) decrease the TNF-α level as compare to HHcy group. However, BEZ235 (4 mg/kg/d, p.o.) and L-NAME (10 mg/kg/d,i.p.,) with high dose of retinoic acid (5 mg/kg/d, p.o.) significantly ($p<0.05$) attenuate the protective effect of retinoic acid.

Discussion

In the present study, hyperhomocysteinemia (>10 µM/l) was produced in Wistar rats by administration of L-methionine. L-methionine administration in dose of 1.7 g/kg/d, p.o. for 28 days causes overload of methionine and hyperhomocysteinemia. Elevated homocysteine concentration has been documented to cause auto-oxidation of the thiol group of Hcy and generate Reactive Oxygen Species (ROS), superoxide anion generation, hydrogen peroxide formation that further degrades nitric oxide and reduces its concentration. Further, these ROS generate other reactive species by inducing excessive lipid peroxidation thus leading to endothelial dysfunction. HHcy has been reported to induce endothelial dysfunction by decreasing the bioavailability of NO and by increasing vascular oxidativestress. Homocysteine has also been documented to stimulate the accumulation of Asymmetric Dimethylarginine (ADMA) that is an inhibitor of eNOS and decreases the formation of nitric oxide [15].

Hyperhomocysteinemia noted in our study has been shown to produce vascular endothelium dysfunction. The degree of ED was assessed by estimating endothelial dependent and independent relaxation in aortic strips. Aorta was isolated and cumulative dose response curve of Ach-induced endothelium-dependent relaxation in phenyl ephrine pre-contracted aortic ring was recorded. The isolated aortic ring preparation is used to avoid the risk of damage to vascular endothelium. Further, sodium nitroprusside-induced endothelium-independent vaso relaxation has been used to investigate the effect of endothelium independent vascular reactivity. Lisinopril (1 mg/kg) has been reported to be a vaso protective drug with pleotrpic actions viz. inhibition of rennin-Ang II-Aldosterone system, increasing nitric oxide production and decreasing oxidative stress thus it is employed as a positive control in this study.

Increased expression of eNOS has been shown to stimulate release of nitric oxide; therefore, reverse transcription polymerase chain reaction has been employed to assess the extent of eNOS expression in aorta. Endogenous formation of nitric oxide is very unstable and gets converted to nitrate and nitrite, therefore, estimation of serum nitrite/nitrate concentration has been used as an index of indirect measure of nitric oxide release [16]. Thus, this parameter has been used as an index to change in nitric oxide formation due to modulation of endothelium function.

As a major regulator of local vascular homeostasis, the endothelium maintains the balance between vasodilatation and vasoconstriction, inhibition and promotion of the proliferation and migration of smooth muscle cells, prevention and stimulation of the adhesion and aggregation of platelets, as well asthrombogenesis and fibrinolysis. Vascular endotheliumreleases nitric oxide which stimulates vaso-relaxation and preserves the integrity of vascular endothelial lining. Exposure of endothelial cells to HHcy led to the formation of S-nitroso-homocysteine, decreasing the bioactivity of NO [17]. Therefore in the present study the observed decrease inexpression of mRNA of eNOS

in aorta, serum concentration of nitrate/nitrite, attenuation of Acetylcholine-induced endothelium-dependent relaxation and increasein TBARS level, decrease in reduced glutathione level in hyperhomocysteinemic rats, may be a consequences of vascular endothelium dysfunction.

Hyperhomocysteinemia leads to the imbalance of phosphorylation and dephosphorylation status of protein kinases that cause modulation of vascular L-arginine/nitric oxide synthase (eNOS), to produce vascular endothelium dysfunction. The increased expression of eNOS and hence increased production of nitric oxide, is important for vascular homeostasis, vessel remodeling and angiogenesis [18]. Retinoic acid employed in present study has been reported to activate PI3K/Akt pathway and up regulate NO synthesis and has a beneficial effect in endothelial cell. Retinoic acid has been documented to stimulate mTOR and inhibit the apoptosis and NF-kB and shows cardiac protection. Thus, a dual inhibitor of PI3K and mTOR- BEZ235 and inhibitor of eNOS, L-NAME were employed in this study.

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine that is documented to be elevated in chronic inflammatory states such as hypertension, diabetes, myocarditis, DCM, heart failure. HHcy increases TNF-α expression, which enhances oxidative stress through up regulating a Nox1 based NAD (P) H oxidase. Thus, TNF-α induces apro inflammatory vascular phenotype in HHcy that potentially contributes to the development of coronary atherosclerosis. Activated tumour necrosis factor-α (TNF-α) produce Reactive Oxygen Species (ROS).

Administration of Retinoic acid (2.5 m/kg and 5 mg/kg) for 1 week (21st to 28th day) to the hyperhomocysteinemic rats, significantly improves the vascular endothelium dysfunction and shows marked reduction in homocysteine level that may be due to decrease in oxidative stress and auto-oxidation of homocysteine assessed in terms increased reduced glutathione level, serum nitrite/ nitrate conc., decreased TBARS level, increased Ach-induced endothelium-dependent vaso-relaxation as compared to hyperhomocysteinemic group. However, SNP did not produce any changes on SNP-induced endothelium-independent vaso-relaxation that shows the effect is specifically due to improvement of endothelium function. Retinoic acid produced PI3K/Akt-dependent phosphorylation of eNOS and increased the generation of nitric oxide and prevented the endothelial dysfunction in hyperhomocysteinemia. Retinoic acid and lisinopril treatment for one week significantly increase the mRNA expression of eNOS and decrease the TNFα levels. Administration of Retinoic acid (5 mg/kg) with L-NAME (10 mg/kg) and BEZ235 (4 mg/kg) significantly blocked the effect of retinoic acid, indicating that PI3K/mTOR and eNOS pathway plays a significant role in retinoic acid signalling.

Thus it may be concluded that retinoic acid improves endothelial dependent vaso-relaxation and attenuates endothelial dysfunction in hyperhomocysteinemic rats possibly by activation of PI3K/mTOR and eNOS pathway.

Conclusion

Thus it may be concluded that retinoic acid improves endothelial dependent vasorelaxation and attenuates endothelial dysfunction in hyperhomocysteinemic rats possibly by activation of PI3K/mTOR and eNOS pathway.

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References

1. Tirziu D, Simons M. Endothelium as master regulator of organ development and growth. Vasc Pharmacol. 2009;50:1-7.
2. Lai WK, Kan MY. Homocysteine-induced endothelial dysfunction. Ann Nutr Metab. 2015;67:1-2.
3. Lentz SR, Rodionov RN, Dayal S. Hyperhomocysteinemia, endothelial dysfunction, and cardiovascular risk: The potential role of ADMA. Atheroscler Suppl. 2003;4:61-65.
4. Malinowska J, Kolodziejczyk J, Olas B. The disturbance of hemostasis induced by hyperhomocysteinemia; The role of antioxidants. Acta Biochim Pol. 2012;59.
5. Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. Annu Rev Pathol. 2011;6:345-364.
6. Uruno A, Sugawara A, Kanatsuka H, Kagechika H, Saito A, Upregulation of nitric oxide production in vascular endothelial cells by all-trans retinoic acid through the phosphoinositide 3-kinase/Akt pathway. Circulation. 2005;112:727-736.
7. Limpach A, Dalton M, Miles R, Gadson P. Homocysteine inhibits retinoic acid synthesis: A mechanism for homocysteine-induced congenital defects. Exp Cell Res. 2000;260:166-174.
8. Jakubowski H. Pathophysiological consequences of homocysteine excess. J Nutr. 2006;136:1741S-1749S.
9. Sastry KV, Moudgal RP, Mohan J, Tyagi JS, Rao G. Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. Anal Biochem. 2002;306:79-82.
10. Dudzinski DM, Michel T. Life history of eNOS: Partners and pathways. Cardiovasc Res. 2007;75:247-260.
11. Kobayashi T, Taguchi K, Nemoto S, Nogami T, Matsumoto T, Activation of the PDK-1/Akt/eNOS pathway involved in aortic endothelial function differs between hyperinsulinemic and insulin-deficient diabetic rats. Am J Physiol Heart. 2009;297:H1767-775.
12. Aukrust P, Ueland T, Lien E, Bendtzen K, Müller F. Cytokine network in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. Am J Card. 1999;83:376-382.
13. Csiszar A, Ungvari Z. Endothelial dysfunction and vascular inflammation in type 2 diabetes: Interaction of AGE/RAGE and TNF-α signaling. Am J Physiol Heart. 2008;295:H475-476.
14. Jacobsen DW, Gatautis VJ, Green R, Robinson K, Savon SR, Secic M, et al. Rapid HPLC determination of total homocysteine and other thiols in serum and plasma: Gender differences and correlation with cobalamin and folate concentrations in healthy subjects. Clin Chem. 1994;40:873-881.

15. Katrusiak AE, Paterson PG, Kamencic H, Shoker A, Lyon AW. Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl–glycine, homocysteine and glutathione in plasma and cell extracts. J Chromatogr B Biomed Appl. 2001;758:207-212.

16. Shah DI, Singh M. Possible role of Akt to improve vascular endothelial dysfunction in diabetic and hyperhomocysteinemic rats. Mol Cell Biochem. 2007;295:65-74.

17. Sharma S, Singh M, Sharma PL. Mechanism of attenuation of diabetes mellitus and hypercholesterolemia induced vascular endothelial dysfunction by protein tyrosine phosphatase inhibition. Vasc Pharmacol. 2011;54:80-87.

18. Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95.