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Homocysteine Excess and Vascular Endothelium Dysfunction: Delineating the Pathobiological Mechanisms

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Abstract: Homocysteine (Hcy) excess as a result of impaired metabolism due to deficiency in cofactors (vitamin B_6, B_{12}, folate) or genetic alteration in metabolic enzymes (Methionine synthase, methyltetrahydrofolate reductase, cystathionine β-synthase and cystathionine-γ-lyase) lead to acquired metabolic anomaly known as hyperhomocysteinemia. Hyperhomocysteinemia (HHcy) is an independent major casual determinant of cerebrovascular and cardiovascular disorders viz., carotid artery disease, atherosclerosis, stroke and vascular dementia. The intense dysfunction of vascular endothelium following hyperhomocysteinemia has been implicated as a factor in the extension of pathological conditions. One of the therapeutic goals of modern vascular biology is to design strategies to limit vascular endothelium dysfunction. A sound understanding of the abnormality in homocysteine metabolic pathway and vascular endothelium dysfunction is necessary before a specific intervention is pursued. Summarized is the description of the homocysteine, its fate and abnormalities that leads to hyperhomocysteinemia. Further, the pathobiology of homocysteine excess which are noted to cause oxidative stress, impairment of intracellular transduction pathways, release of inflammatory mediators, dysfunction of endothelial progenitor cells and RBCs hemolysis is also discussed. Collectively these pathobiological mechanisms causes the decrease in cell survival, activation of proinflammatory mediators, apoptosis and consequently leads to vascular endothelium dysfunction. On the basis of the multiple toxic effects of homocysteine excess, interventions designed for these mechanisms may provide novel targets for the development of vascular protective agents.

Key words: Hyperhomocysteinemia, genetic alteration, oxidative stress, phosphatidylinositide 3-kinases, protein kinase B pathway, vascular endothelium dysfunction

INTRODUCTION

Homocysteine is a naturally occurring nonproteogenic sulfur-containing amino acid produced as part of the body's methylation process. The Hcy accumulated either in inherited disorders, that alters enzyme activity in metabolic transsulfuration and remethylation pathways or alternatively, in nutritional deficiencies of essential cofactors or enzyme substrates, including cobalamine (vitamin B_{12}), folate or pyridoxine (vitamin B_6), thus results in blockage of homocysteine metabolic pathways (Ingenbleek, 2011). Low level of homocysteine (Hcy) (5-14 µmol L^{-1}) is normally found in the plasma (Sharifi et al., 2010). Homocysteine levels can range from a low level of 14 µmol L^{-1} to a devastating level of 100 µmol L^{-1} in severe cases (Seshadri et al., 2002). Hyperhomocysteinemia (HHcy) is an acquired metabolic anomaly which was first identified by McCully (Ingenbleek, 2011). The HHcy may leads to cardiovascular and cerebrovascular complications like atherosclerosis, thrombosis, stroke, impairs angiogenesis, Alzheimer's disease, dementia, declining memory, poor concentration and judgment and lowered mood etc. Increase in homocysteine concentration is also crucially involved in various autoimmune and malignant diseases (Dalton et al., 1997). Hyperhomocysteinemia impairs vascular function and is a putative risk factor for cardiovascular and cerebrovascular diseases in underprivileged malnutrition population. The HHcy downregulates nitric oxide (NO) bioavailability by upregulating endogenous asymmetric dimethylarginine (ADMA) (Singh et al., 2009) which is an endogenous endothelial nitric oxide synthase (eNOS) inhibitor, thus results in vascular endothelial dysfunction. High levels of homocysteine may lead to increased production of oxidation products, homocysteine thiolactone and homocysteine mixed disulfides which may damage endothelium by excessive sulfation of connective tissue (Obeid and Herrmann, 2006).

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Homocysteine damage the vascular endothelium which is vital part of all blood vessels performing major functions including the control of microvascular permeability, coagulation, inflammation, vascular tone as well as the formation of new vessels via vasculogenesis and angiogenesis in normal and diseased states, respectively (Endemann and Schiffrin, 2004). Damage of the blood vessels leads to Vascular Endothelial Dysfunction (VED). It is characterized by shift of endothelial actions towards reduced vasodilation, proinflammatory and prothrombic properties (Potenza *et al.*, 2009). The modulation of vascular L-arginine/nitric oxide synthase system is hallmark of vascular endothelial dysfunction. The VED results in reduced activation of endothelium nitric oxide synthase (eNOS) and increased production of reactive oxygen species (ROS) which account for reduced synthesis and bioavailability of nitric oxide (Ulker *et al.*, 2003) which is required for the proper working of blood vessels. The dysregulation of endothelial function upregulates the expression of procoagulant, prothrombotic and pro-inflammatory mediators. In the milieu of cardiovascular risk factors that disturb vascular homeostasis, the endothelium becomes dysfunctional resulting in enhanced production of cytokines and expression of cellular adhesion molecules by the endothelium. Various experimental evidences revealed that reduced nitric oxide production and increased oxidative stress lead to VED.

Source and generation of homocysteine: Homocysteine is generated by intra hepatic transmethylation of dietary methionine which is obtained from eggs, sesame seeds, Brazil nuts, fish, meats andcerealgrains (Ingenbleek, 2011). Methionine is converted to the methyl donor S-adenosylmethionine (SAM) by an enzyme methionine adenosyltransferase. The SAM is demethylated to S-adenosylhomocysteine (SAH) by an enzyme SAM-methyltransferase. The SAH is subsequently cleaved into adenosine and homocysteine (De Koning *et al.*, 2003) (Fig. 1).

Metabolism of homocysteine: The metabolism of homocysteine involves two pathways i.e., remethylation pathway and transsulfuration pathway (Ingenbleek, 2011). Normally, either homocysteine is converted into methionine following remethylation (RM) pathways by transfer of a methyl group from 5-methyl tetra hydrofolate catalyzed by methionine synthase, an enzyme that requires vitamin B_{12} as a cofactor, to form methionine (Pandey and Pradhan, 2010) or is catabolized along the transsulfuration cascade involving the irreversible

conversion of homocysteine to cysteine (Ingenbleek, 2011; Selhub, 1999). Transsulfuration reaction involves cystathionine β-synthase (CβS), an enzyme dependent on vitamin B_6 (pyridoxal-5-phosphate) that condenses Hcy with serine to form cystathionine (CTT) which is further converted into cysteine through vitamin B_6 (pyridoxal-5-phosphate) dependent cystathionine-γ-lyase (CγL). Cysteine, thus formed generate various products like glutathione, an important intracellular antioxidant and hydrogen sulfide (H_2S), a gas that can induce endothelial-dependent relaxation and is also converted into sulfates which are excreted in urine. Cysteine can be further converted to cysteine sulfinic acid which is then converted to taurine. Taurine can decrease methionine concentration by decreasing its uptake from intestine, thus normalizing hyperhomocysteinemia. Taurine also reduces the hyperhomocysteinemia induced oxidative stress and also restores the function of extracellular superoxide dismutase thus ameliorate coronary artery wall's pathology by its favorable effect on total plasma homocysteine and apoptosis (Wesseling *et al.*, 2009). Excess of homocysteine is also remethylated by the enzyme betaine homocysteine methyltransferase (BHMT) which transfers a methyl group to homocysteine via demethylation of trimethylglycine (betaine) to dimethylglycine (De Koning *et al.*, 2003) (Fig. 1).

Causes of hyperhomocysteinemia a metabolic anomaly
Deficiency of vitamins: B-vitamins are essential cofactors in both of these Hcy metabolizing pathways. An inadequate dietary intake of nutritional factors such as B-vitamins (Quadri *et al.*, 2004) results in deficiency in all of these coenzymes viz., vitamin B_{12}, folate and vitamin B_6 thus leads to impaired metabolism of Hcy and elevated homocysteine level. Vitamin B_6 (Pyridoxal-5-phosphate) shortage significantly triggers upstream accumulation of Hcy in biological fluid whereas folate or B_{12} deficiency results in downstream sequestration (Ingenbleek, 2011).

Genetic alteration of metabolic enzymes: Genetic alteration results in decrease in expression of Hcy metabolizing enzymes viz., cystathionine β-synthase (CβS), methylenetetrahydrofolate reductase (MTHFR) and Methionine Synthase (MS) that leads to hyperhomocysteinemia (Faraci and Lentz, 2004). The MTHFR is the rate-limiting enzyme in the methyl cycle and it is encoded by the MTHFR gene (Goyette *et al.*, 1994). Mutations in this gene are associated with MTHFR deficiency that may increase the susceptibility to occlusive vascular disease,colon cancer, acute leukemia and neural tube defects (Fodinger *et al.*, 2000). Higher homocysteine level has been found in an individual with
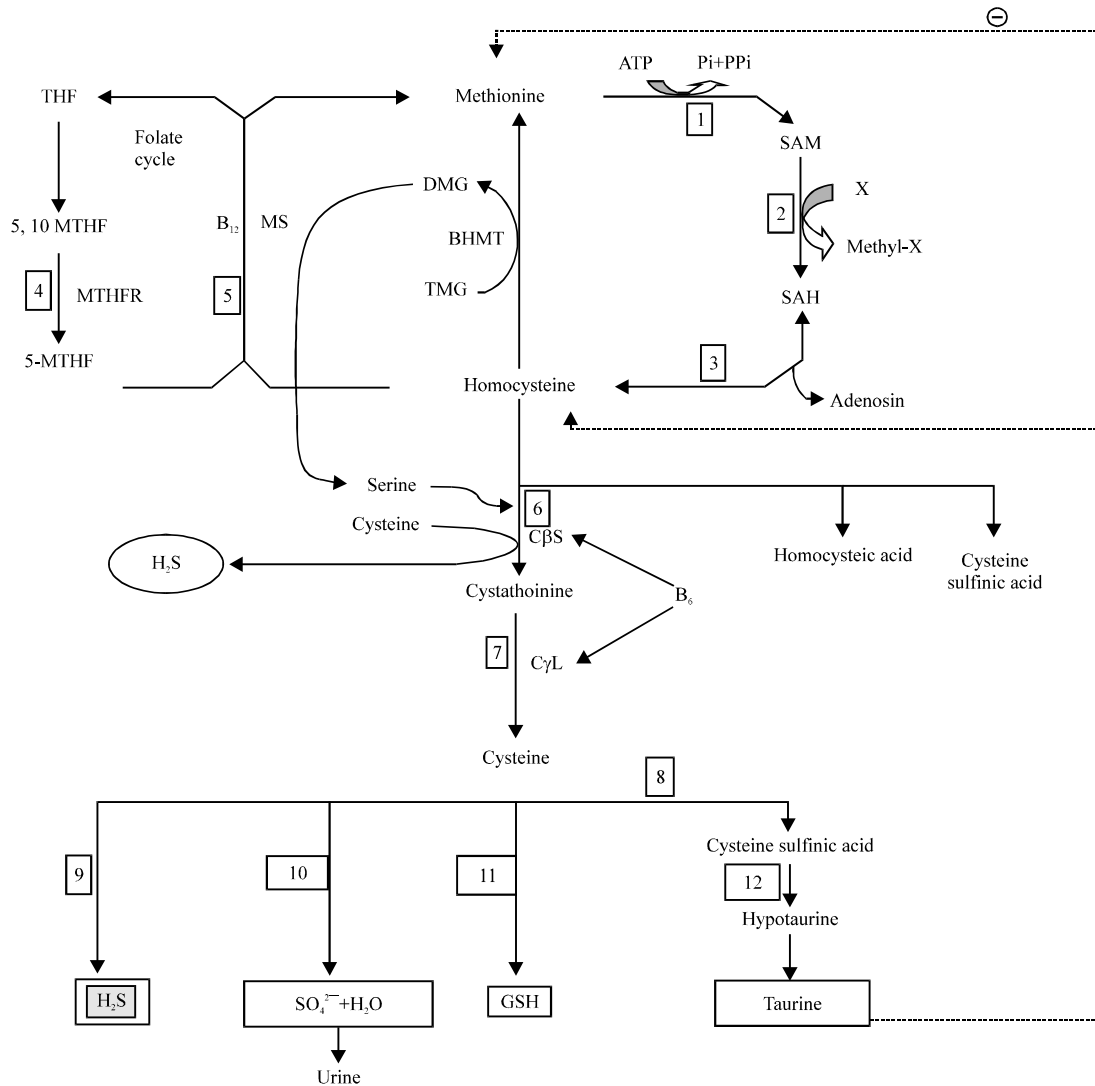
Fig. 1: Schematic representation of the methionine cycle and homocysteine degradation pathways and their toxic metabolites, ATP: Adenosinetriphosphate, THF: Tetrahydrofolate, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, Cysta: Cystathionine, Cys: Cysteine, GSH: Glutathione, H_2S: Hydrogen sulfide, Tau: Taurine, SO_4^{2-}: Sulfate oxyanions, TMG: Trimethylglycine and DMG: Dimethylglycine, Enzymes: (1) Met-adenosyltransferase, (2) SAM-methyltransferase, (3) Adenosyl-homocysteinase, (4) Methylene-THF reductase, (5) Met-synthase, (6) CβS indicates cystathionine-β-synthase, (7) CγL indicates cystathionine-γ-lyase, (8) oxidase, (9) CDO indicates cysteine-dioxygenase, (10) γ-glutamyl-synthase, (11) Reductase, (12) Sulfinoalanine decarboxylase, BHMT indicates betaine homocysteine methyltransferase

C677T genotype than those with the normal C677C genotype (Lea *et al.*, 2009). The C677T polymorphism in the MTHFR enzyme leads to elevated plasma Hcy level (Sottilotta *et al.*, 2010). The MTHFR C677T allele responsible for change in an amino acid sequence which results in diminution in MTHFR enzyme activity leads to mild hyperhomocysteinemia (Frosst *et al.*, 1995). Both the homozygous (TT) and heterozygous (CT)

genotypes are associated with lower tissue concentrations of folate, higher homocysteine concentrations and lower enzyme activity than the wild type (CC) genotype possibly resulting in vascular disease while these effects are more marked in homozygotes (Kirke *et al.*, 2004). The human MTHFR gene mapped to chromosome 1p36.3 catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)

dependent conversion of 5,10-methylenetetrahydrofolate (CH_2-THF) to 5-methyltetrahydrofolate (CH_3THF), the principal circulatory form of folate and a cofactor for methylation of homocysteine to methionine. Individual with MTHFR C677T genotype, don't effectively convert folic acid to its active form (Lea *et al.*, 2009) and thus leads to folic acid (B-complex vitamins) deficiency with subsequent hyperhomocysteinemia which raised the risk factors for vascular dysfunction (Liu *et al.*, 2010).

PATHOBIOLOGICAL CONSEQUENCES OF HYPERHOMOCYSTEINEMIA

Hyperhomocysteinemia and Vascular endothelium oxidative stress: The HHcy leads to the production of Reactive Oxygen Species (ROS) viz., superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), hydrogen peroxide (H_2O_2), nitric oxide free radical ($NO^\bullet$), peroxynitrite radical ($ONOO^-$) and decreases activity of antioxidants such as reduced GSH, superoxide dismutase etc. Excess Hcy leads to the formation of various ROS by following pathways:

- Auto oxidation pathway
- Indirect pathway

In case of autooxidation pathway, thiol group of homocysteine readily undergoes auto-oxidation in plasma to generate H_2O_2 free radical (ROS) (Lawrence *et al.*, 2003).

In indirect pathway, indirect generation of O_2^- from uncoupled eNOS, xanthine oxidase, or NAD(P)H oxidase (NOX) by oxidative stress.

Excess amount of H_2O_2 and O_2^- (generated via autoxidation) free radicals reacts with each other in presence of Cu^{2+} and Fe^{2+} to produce $OH^\bullet$ radical (Miller *et al.*, 2006). The $OH^\bullet$ radical thus formed leads to oxidation of Low Density Lipoproteine (LDL) to form lipid peroxyl radical ($LOO^\bullet$). Previously formed O_2^- and $LOO^\bullet$ react with endothelium derived Nitric Oxide (NO) to produce peroxynitrites ($ONOO^-$) and lipidperoxynitrite ($LOONO^\bullet$) radicals, respectively.

ROS are potentially toxic by-products of cellular metabolism. When generated in excess, ROS can react rapidly with biomolecules causing cellular damage such as DNA mutations, lipid peroxidation and protein oxidation (Miller *et al.*, 2006). The ROS leads to depletion of intracellular glutathione by the downregulation of antioxidant enzymes (Fig. 2).

Endogenous H_2O_2 free radicals generally elicits cerebral vasodilatation by opening of potassium channels while its higher concentrations causes vasoconstriction followed by vasodilatation (Miller *et al.*, 2006).

Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are known to be strong inducers of nuclear factor kappa-B (NFκ-B) activation that belong to the family of transcription factors regulate the induction and resolution of inflammation (Sharma and Singh, 2012). The NFκ-B regulates the expression of genes, including those encoding adhesins and cytokines (e.g., TNF-α and interleukin-1) (Taoufiq *et al.*, 2008) thus, thought to promote cardiovascular disorder including endothelial dysfunction through its pro-inflammatory, pro-adhesion and pro-oxidant gene transcription. Activation of NFκ-B leads to aging induced dementia as a result of upregulation of genes of pro-inflammatory enzymes. The NFκ-B may also decrease the acetylcholine activity by increasing acetylcholinesterase (AchE) which leads to cognitive deficits and vascular impairment (Sharma and Singh, 2012).

Peroxynitrite ($ONOO^-$) (formed by the reaction of $NO^\bullet$ with superoxide) is increased in peripheral blood vessels during HHcy and known to activate poly (ADP-ribose) polymerase (PARP) which is an important mediator of vascular dysfunction in disease states such as diabetes (Faraci and Lentz, 2004). The PARP activation may promote the activation of NF-κB, MAP kinases and the expression of proinflammatory mediators, adhesion molecules such as ICAM-1 and iNOS (Virag and Szabo, 2002) which subsequently effects vascular endothelial cells function. Peroxynitrite also inhibits PI3K that further reduces eNOS phosphorylation, therefore, lowering NO bioavailability which is the major consequences of vascular endothelial dysfunction (Bao *et al.*, 2010) (Fig. 3).

Additionally, Peroxynitrite ($ONOO^-$) can also produce vascular endothelial dysfunction and oxidative stress by nitration of tyrosine residues in the mitochondrial isoform of superoxide dismutase (Mn-SOD) and by possibly promoting the uncoupling of NO synthases, a circumstance in which the normal flow of electrons within NO synthase is diverted to produce superoxide anion (O_2^-) rather than NO (Faraci and Lentz, 2004).

Another effect of HHcy has been reported to increase in ROS production in endothelial cells of blood vessels through NADPH oxidase activation (Carluccio *et al.*, 2007). The HHcy increased NADPH oxidase activity leads to the increase in intracellular superoxide. The NADPH
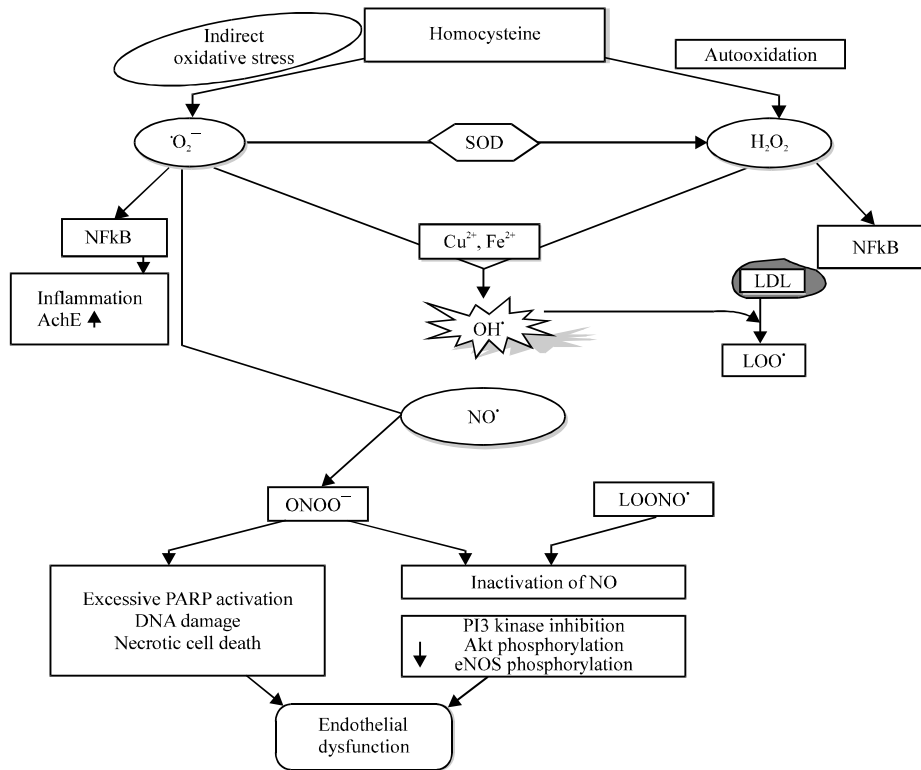
Fig. 2: Homocysteinl and oxidative stress related endothelial dysfunction, Superoxide anion (O_2^-), Hydrogen peroxide (H_2O_2), Hydroxyl radical ($OH^\bullet$), Lipidperoxyl radical ($LOO^\bullet$), Nitric oxide free radical ($NO^\bullet$), Peroxynitrite radical ($ONOO^-$), Lipid peroxynitrite radical (LOONO), Superoxide dismutase (SOD), Hydrogen sulfide (H_2S), Nuclear factor kappa-B (NFκ-B), Acetylcholinesterase (AchE)
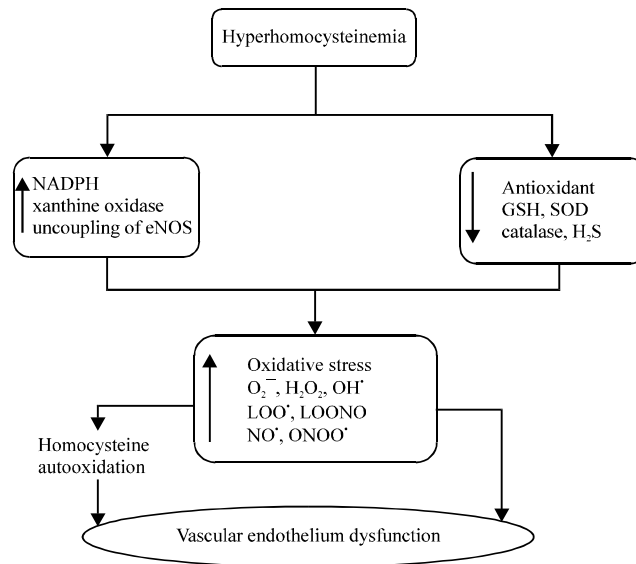

Fig. 3: Hyperhomocysteinemia and oxidative stress related endothelial dysfunction, O_2^-: Superoxide anion, H_2O_2: Hydrogen peroxide, $OH^\bullet$: Hydroxyl radical, $LOO^\bullet$: Lipidperoxyl radical, $LOONO^-$: Lipid peroxynitrite radical; $NO^\bullet$: Nitric oxide free radical, $ONOO^-$: Peroxynitrite radical, GSH: Reduced glutathione, SOD: Superoxide dismutase, H_2S: Hydrogen sulfide, NFκ-B: Nuclear factor kappa-B

oxidase (Nox) is a transmembrane/cytosolic multi-subunit enzyme that transfers electrons from NADPH to molecular oxygen to produce superoxide. Superoxide produced by Nox can spontaneously form hydrogen peroxide (H_2O_2) which undergoes further reactions to generate ROS. However, Nox is composed of multiple cytosolic regulatory components (p47phox, p67phox, p40phox and rac) and a transmembrane catalytic heterodimer (p22phox and gp91phox). When Nox is activated, regulatory cytosolic components are translocated to cellular membranes, where they form a functional complex that generates superoxide radicals. The Nox family of NADPH oxidases has seven members, including Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Bao *et al.*, 2009). The NADPH oxidase (Nox4) has been located in the nucleus of endothelial cells and it has been suggested that it might regulate gene expression and activate a serine/threonine protein kinase through the production of ROS (Bao *et al.*, 2009).

Moreover, HHcy increases TNF-α expression which enhances oxidative stress through upregulating a Nox1 based NAD(P)H oxidase. Thus, TNF-α induces a proinflammatory vascular phenotype in HHcy that potentially contributes to the development of coronary atherosclerosis (Ungvari *et al.*, 2003). It is hypothesized that in HHcy, TNF-α upregulates NAD(P)H oxidase-dependent O_2^- production both by induction of the oxidase subunit Nox1 and an increased phosphorylation of the regulatory subunits. HHcy also induces VCAM-1 gene expression through NAD(P)H oxidase activation. The VCAM-1 expression is crucially involved in the monocyte adhesion and early atherogenesis in the vascular wall (Carluccio *et al.*, 2007). Excess of Hcy decreases the binding of extracellular superoxide dismutase (EC-SOD) to vascular endothelial cell surfaces by degradation of endothelial heparan sulfate proteoglycan which results in a loss of the ability to protect endothelial cell surfaces from oxidative stress. The EC-SOD in the vascular system diffuses into the extracellular space and scavenges superoxide before it reacts with NO. Elimination of EC-SOD from the endothelium may result in the elevation of superoxide which would then produce highly reactive peroxynitrite ($ONOO^-$) (Yamamoto *et al.*, 2000). Therefore, it has been suggested that HHcy decreases the ability to scavenge superoxide and indirectly causes injury of the vascular endothelium.

HHcy also impairs the glutathione peroxidase activity (Handy *et al.*, 2005) thus sensitizing cells to the cytotoxic effects of agents or conditions known to generate ROS.

Catalase or glutathione peroxidase normally metabolizes H_2O_2 into water. So decrease in activity of these enzymes may lead to non-enzymatic conversion of H_2O_2 into OH^- by reaction with heavy metals (Fe^{2+}) (Miller *et al.*, 2006) (Fig. 2).

Furthermore, oxidative stress accounts for the initiation of apoptosis and the dysfunction of EPCs induced by HHcy. Impairment of NO-mediated vascular protection is also related to the effects of Hcy on endothelial progenitor cells (EPCs) (Bao *et al.*, 2010).

Hyperhomocysteinemia and vascular endothelium interacellular pathways: The HHcy impairs the cellular and molecular mechanisms of vascular endothelial cells by causing imbalance between phosphorylation and dephosphorylation state of lipid and protein kinases which modulates vascular L-arginine/nitric oxide synthatase activity and may produce vascular endothelium dysfunction. The HHcy has been reported to impair Phosphatidylinositide 3-kinases/Protein Kinase B (PKB) [PI3K/Akt] pathway by activation of protein tyrosine phosphatase (PTPase) (Sharma *et al.*, 2011a) and Rho kinase mediated intracellular signaling pathway (Park *et al.*, 2011).

Activation of PI3K/Akt pathway mediates the rapid increase in eNOS activity (Sharma *et al.*, 2011b) thus increasing the availability of NO. Overactivation of PTPase and decrease in PI3K/Akt pathway is implicated in the regulation of cellular proliferation, differentiation and apoptosis by its action to enhance proapoptotic genes such as Caspase, BCL-2 and Bcl-2-associated death promoter (BAD) (Sharma *et al.*, 2011a). The PTPase also attenuates various growth factors or extracellular matrix (Meredith *et al.*, 1993) and inactivates Mitogen-Activated Protein Kinases (MAP Kinases) which leads to endothelial cells apoptosis (Harrington *et al.*, 2000). Inhibition in PTPase activity have been reported to attenuate hyperhomocysteinemia induced vascular and cardiac complications (Harrington *et al.*, 2000).

Rho-kinase is reported to be involved in vascular endothelial dysfunction. Rho kinase is a small GTP-binding protein involved in intracellular signaling pathway. Hyperhomocysteinemia leads to abnormal activation of Rho kinase pathway (Taoufiq *et al.*, 2008) which likely to be acts upstream of PI3K/Akt (Park *et al.*, 2011). Rho-kinase also increases the generation of ROS (Brown *et al.*, 2006) which reduces the biosynthesis of NO (Kumar *et al.*, 2007; Nohria *et al.*, 2006). Rho kinase also increases the vascular endothelium permeability (Clements *et al.*, 2005) by modulating the phosphorylation

of myosin light chain as well as tight junctions (Taoufiq *et al.*, 2008). Rho kinase can also activate various cascades through its ability to phosphorylate multiple substrates (including other kinases), thereby increasing NFκ-B activity (Taoufiq *et al.*, 2008) that leads to vascular impairment. Inhibition of Rho-kinase by fasudil leads to the rapid phosphorylation and activation of Akt via PI3K, resulting in an increase in NO production (Sharma *et al.*, 2011b) and improving hyperhomocysteinemia induced vascular endothelial dysfunction (Shah and Singh, 2006a).

Hyperhomocysteinemia is involved in the production of high oxidative stress which has been reported to activate Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. c-Jun N-terminal kinases (JAKs) family consists of JAK1, JAK2, JAK3 and tyrosine kinases-2 (Tyk2) (Marrero *et al.*, 1995). Increase in activation of protein tyrosine phosphataseshas been documented to remove phosphates from cytokine receptors and activate STATs (Hebenstreit *et al.*, 2005). Thus, activated JAK pathway has been demonstrated to inhibit PI3K/AKT pathway (Faraci and Lentz, 2004).

Proteine Kinase A (PKA) activation has been reported to improve hyperhomocysteinemia induced vascular endothelium dysfunction (VED) by modulating various intracellular pathways. It is reported that 8-Br-cAMP, an activator of PKA ameliorates hyperhomocysteinemia induced VED. The cAMP-dependent protein kinase A is the principle intracellular target for cAMP in mammalian cells

(Shah and Singh, 2006b). The PKA activation stimulates phosphorylation of eNOS and opening of ATP sensitive K^+ channels which leads to activation of eNOS and thus increases the bioavailability of NO (Boo and Jo, 2003). The PKA also attenuate Rho-kinase associated inhibition of eNOS via PI3K/Akt pathway (Kumar *et al.*, 2007; Boo and Jo, 2003), PKA also reduces the hyperhomocysteinemia induced generation of ROS (Sullivan *et al.*, 2001) and mediates the Bradykinin induced production of endothelial NO (Bae *et al.*, 2003) thereby, decreasing the permeability of endothelium and apoptotic death of endothelial cells (Irie *et al.*, 2001; Kulhanek-Heinze *et al.*, 2004) thus ameliorating hyperhomocysteinemia related vascular endothelial dysfunction (Fig. 4).

Hyperhomocysteinemia and vascular endothelium progenitor cells: Progenitor cells are primitive Bone Marrow (BM) cells that are located at the periphery of the blood islands and have the capacity to proliferate, migrate and differentiate into mature endothelial lineage cells (Asahara and Kawamoto, 2004). Endothelial Progenitor Cells (EPCs) are derived from more differentiated CD34 or immature CD133 hematopoietic stem cells and blood mononuclear cells or CD14 monocytes. The EPCs maintain vascular homeostasis by restoring an intact endothelium (Kruman *et al.*, 2000). The EPCs provides a circulating pool of cells that could form a cellular patch at the site of denuding injury and thus act as cellular reservoir to
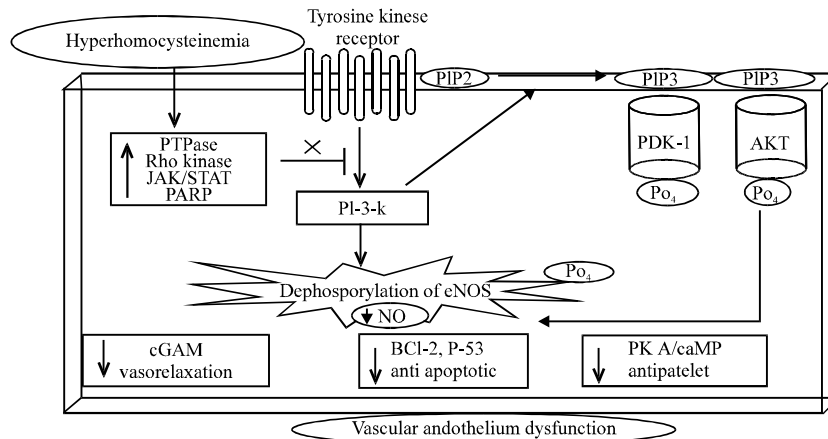

Fig. 4: Vascular endothelium cell intracellular PI3K/PDK/Akt pathway attenuated by hyperhomocysteinemia due to increase in protein tyrosine phosphatase, Rho kinase, JAK/STAT and PARP that leads to vascular endothelium dysfunction. PTPase: Protein tyrosine phosphatase, JAK/STAT: Janus kinase/signal transducer and activator of transcription, PARP: Poly (ADP-ribose) polymerase, PI3K: Phosphatidylinositide 3-kinases, Akt: Protein Kinase B (PKB), PIP2: Phosphatidylinositol 4,5-bisphosphate, PDK-1: Phosphoinositide dependent protein kinase-1, cGMP: Cyclic guanosine monophosphate, PKA: Protein kinase A

replace dysfunctional endothelium. Impaired mobilization or depletion of these EPCs contributes to endothelial dysfunction (Hill *et al.*, 2003). Hyperhomocysteinemia (HHcy) reduces Endothelial Progenitor Cell (EPC) numbers and impairs its functional activity (Zhu *et al.*, 2006) which is associated with EPCs senescence. The HHcy accelerates the onset of EPCs senescence through telomerase inactivation or by decreasing the Akt phosphorylation leading to cellular dysfunction (Zhu *et al.*, 2006; Xia *et al.*, 2008). Telomerase acts by delay or decreasing endothelial cell senescence by Akt dependent manner. Akt acts by increasing the activity of the eNOS, thereby, increasing the availability of NO that has been demonstrated to activate telomerase and reprieves endothelial cell senescence. The HHcy mediated EPCs apoptosis is also associated with caspase-8, cytochrome-c release and caspase-3 activation. HHcy induced increased ROS, acting as an upstream factor for mitochondrial membrane depolarization, forces mitochondria to release cytochrome-c and caspases, resulting in eventual cellular apoptosis and dysfunction (Bao *et al.*, 2010) (Fig. 5).

Immune activation, Hyperhomocysteinemia and vascular endothelium proinflammatory mediators: One of the major cause of HHcy is the activation of immune system. Most diseases associated with hyperhomocysteinemia are due to immune system activation (Erren *et al.*, 1999; Rogers *et al.*, 1996). Chronic activation of the immune system leads to the enhanced production of oxidative stress which may be the major pathogenic mechanisms contributing to the development and progression of diseases such as atherosclerosis, vascular dementia etc. Many factors such as aging, injury, cancer, autoimmunity etc. are reported in literature that stimulates immune system which further activates immunocompetent T-cells and monocytes. Proliferated T-cells (Th1-type immune activation) release Interferon-γ (IFN-γ) and Hcy. The IFN-γ further stimulates monocytes to release ROS (Schroecksnadel *et al.*, 2003) whereas, Hcy get accumulated and start cascade of producting ROS. ROS thus formed activates nuclear factor kappa-B (NFk-B) which enhances cytokine production eg. tumor necrosis factor-α (TNF-α) (Lazzerini *et al.*, 2007) and also upregulates the expression of various adhesion molecules such as vascular cell adhesion molecule (VCAM-1) and E-selectin, P-selectin (Silverman *et al.*, 2002) with subsequent inflammatory responses and endothelial dysfunction (Pruefer *et al.*, 1999) (Fig. 5). The NFk-B activation modifies the expression of Monocyte Chemoattractant Protein-1 (MCP-1) (Wang *et al.*, 2000). MCP-1 is a potent chemokine (Nelken *et al.*, 1991) that together with adhesion (molecules VCAM-1 and E-selectin) bind to circulating monocyte/macrophage, leading to cell attachment and migration into the sub-endothelial space (Wang *et al.*, 2002) which activates inflammatory cascade (Li and Glass, 2002). It clearly shows that HHcy is the interlink between the immune system and adhesion molecules (Schroecksnadel *et al.*, 2003).

Enhanced ROS also initiates oxidation of Low-Density Lipoprotein (LDL) to produce Modified Low Density Lipoprotein (MLDL). These MLDL is taken up by macrophages and ending up with the formation of foam cells (Schroecksnadel *et al.*, 2003). Foam cells are not
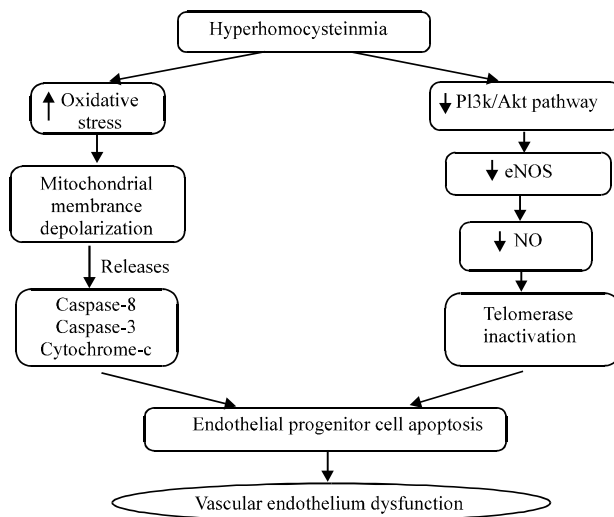

Fig. 5: Endothelial progenitor cell apoptosis and vascular endothelium dysfunction by hyperhomocysteinemia
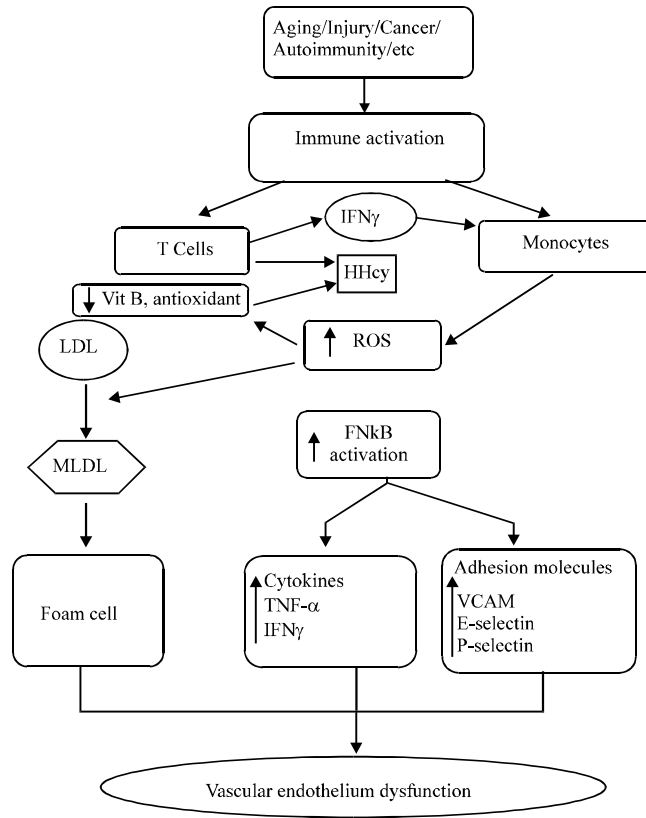
Fig. 6: Hyperhomocysteinemia and immune cell activation in the development of atherosclerosis. ROS: Reactive oxygen species, LDL: Low-density lipoprotein, MLDL: Modified low-density lipoprotein, NFk-B: Nuclear factor kappa-B, TNF-α: Tumor necrosis factor-α, INF-γ: Interferon gamma, VCAM: Vascular cell adhesion molecule

dangerous as such but can become a problem when they accumulate at particular site of the blood vessels. It creates a necrotic centre ofatherosclerosis which obstructs the adequate amount of blood to flow resulting inischemia and contributing to stroke and myocardial infarction which are the two leading causes of cardiovascular death related to hyperhomocysteinemia (Fig. 6).

Hyperhomocysteinemia, red blood cells and vascular endothelium dysfunction: The 5-MTHF transfers its methyl group to Hcy and converted into methionine and THF (Fig. 1). This reaction requires vit B_{12} as a cofactor. Deficiency of vit B_{12} and mutation in enzyme MTHFR C677T allele (Ventura *et al.*, 2004) leads to accumulation of Hcy which further causes the RBCs haemolysis.

Homocysteine has been proposed as a hemolytic toxin (Acharya *et al.*, 2008). The HHcy cause vascular endothelium dysfunction consequence of which leads to immediate microangiopathy (A disease of the capillaries) initiating the lysis of the RBCs (Acharya *et al.*, 2008). The

HHcy activates polymorphonuclear leukocytes which increases hemolytic activity of RBCs (Olinescu *et al.*, 1996). It has been well documented in literature that homocysteine down regulates the cellular glutathione peroxidase-1 activity that increases the production of reactive oxygen species which may subsequently provoke the oxidative vulnerability of sulfhydryl groups of hemoglobin leading to hemoglobin precipitation within the RBC and induces cell lysis.

Hyperhomocysteinemia also stimulates NMDA receptors (present on the erythrocytic membrane) which induce the Ca^{2+} intake in the RBCs (Boldyrev, 2010) thus leads to RBCs aggregation and structural deformation causing hemolysis (Muravyov and Tikhomirova, 2012). Moreover, infected erythrocytes adhere to the endothelial cells can directly trigger the Rho kinase activity thereby may block the PI3K/Akt pathway mediated eNOS activation (Taoufiq *et al.*, 2008) and results in increased production of Reactive Oxygen Species (ROS) by endothelial cells, leading to vascular endothelium dysfunction or cell death (Pino *et al.*, 2003; Taoufiq *et al.*, 2006) (Fig. 7).
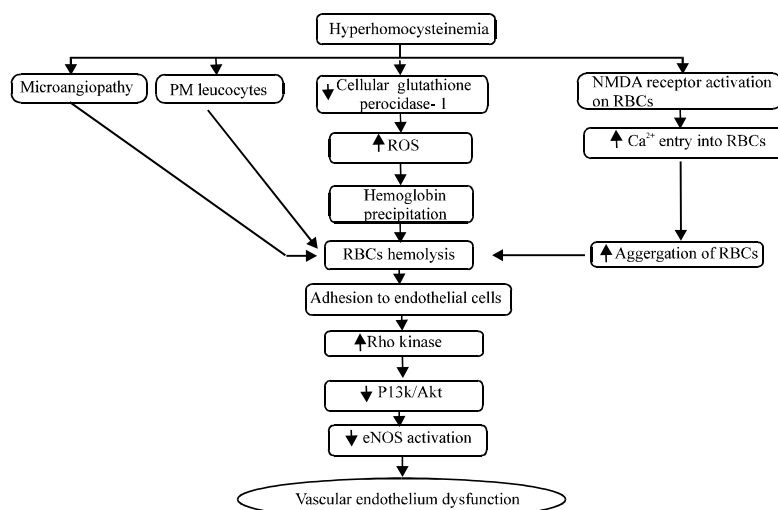
Fig. 7: Hyperhomocysteinemia induced vascular nedothelium dysfunction by RBC hemolysis. PMN: Polymrophonuclear

CONCLUSION

It has been clear that mutation in MTHFR genes, deficiency of vitamin B_{12}, folate, betaine and B_6 as a result of malnutrition, normal aging and diseased conditions are the main causes for increased homocysteine level. Despite the fact that there have been major advances in the identification of HHcy as an important independent metabolic anomaly, molecular regulators in disease progression and its role in the various pathological conditions remains to be identified. Better understanding of pathology of HHcy may provide interventions in the regulation and progression of several vascular disorders such as dementia, ischemia, cardiovascular diseases and haemostatic disorders. Vascular endothelium dysfunction is the first event that is followed by smooth muscle and basement membrane abnormalities that leads to insufficient blood supply and exaggerate vascular pathology. Thus further studies are warranted to translate this scientific knowledge into potential pharmacological interventions for HHcy and its associated vascular disorders.

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