Review of Literature

The impact of "reperfusion injury" related to ischemic myocardial and cerebral conditions is enormous and this enormity of the cerebraovascular and cardiovascular diseases has driven the scientific world in the pursuit of the ideal therapy or strategy to restrict the inflicted damage. Coronary heart diseases and coronary heart diseases take heavy toll on the population of lower and middle income group countries. These countries bear 75% of the CVD. The scarcity of data makes more difficult to analyze the actual extent of these diseases in the developing countries. The major reason for the slow and agonizing progress towards developing an intervention to treat these pathological complications is the absence of strategy for secondary prevention in CVD and CHD[9]. We have designed the current study to analyze the approach of combination of various pharmacologically active drugs. This approach gives us the liberty to study the effect of two different active drugs acting independently or synergistically in the combination.

To start with, the extensive review of the mechanisms of injury involved in myocardial and cerebral ischemia/reperfusion injury was carried out. Various factors assisting the ischemic damage were discussed in detail.

2.1 Myocardial ischemia/reperfusion injury:

Globally, ischemic heart diseases will remain one of the major causes of human death will into the 21st century. A Roman stoic philosopher, Seneca had written 2000 years ago, probably the first recognized description of myocardial ischemia, that "The attack is very short, and like a storm. It usually ends within an hour to have any other malady is to be sick, to have this is to be dying" [55].

Myocardial ischemia initiates a continuum of progressively more severe cellular changes that, unless interrupted by early reperfusion, inevitably culminate in cell death and tissue necrosis. Following the total or partial occlusion of a coronary artery, metabolic and functional changes are initiated within seconds. These changes, which become progressively more severe with time, are initially of a reversible nature. However, with increasing durations of ischemia, they become more severe and eventually irreversible injury occurs[56]. Proximal coronary occlusion of upto 15 min results in reversible injury, beyond this cutoff time, the injury may be irreversible[57,58]. This brief period (15 min) of ischemia, however, induces numerous changes in the noncontracting myocytes, including a marked decrease in high-energy phosphates and the H^+, and mild

intracellular edema observed on ultrastructure; but once blood flow is reestablished, the myocytes eventually recover[58]. These brief episodes of ischemia influence the myocardium in both positive and negative ways and that this influence may last for days. Brief episodes of ischemia induce both stunned myocardium and ischemic preconditioning. Myocardial stunning is the best-established manifestation of reperfusion injury[4,7]. It is defined as "prolonged postischemic dysfunction of viable tissue salvaged by reperfusion,"[57,58]. Ischemia is often accompanied by reperfusion and reperfusion can not occur without prior ischemia. The reperfusion is essential for survival of tissue/cell, but it carried a risk of extending the cell damage as a result of reperfusion itself. Thus, there exists a paradox in that the tissue viability can be maintained only if reperfusion is instituted within a reasonable time period, but only at the risk of extending the injury beyond that due to the ischemic insult itself. This paradox is again conformed by the initiation of ventricular fibrillation during the reperfusion[59]. Myocardial reperfusion injury is defined as the death of myocytes, which were alive at the time of reperfusion, as a result of one or more events initiated by reperfusion. Thus the damage to the myocardial cells is caused due to the restoration of blood flow. This reperfusion injury can also be states as "those metabolic, functional, and structural consequences of restoring coronary arterial flow can be avoided or reversed by the modification of the conditions of reperfusion"[60]. The duration of the ischemic milieu is quite crucial for the development of necrotic cell death. Tissue necrosis originates in the subendocardial regions of the ischemic myocardium and extends to the subepicardial regions of the area at risk, often termed as the wave-front phenomenon. The reperfusion injury induced cell death is characterized by microscopic observations of explosive swelling, which includes disruption of the tissue lattice, contraction band, mitochondrial swelling, and calcium phosphate deposits within mitochondria[55,61].

Briefly, reperfusion injury mechanism involves the events occurring during ischemia and reperfusion. Ischemia precludes adequate oxygen supply, which results in rapid depletion of adenosine triphosphate (ATP). This inhibits ATP driven Na^+- K^+ pumps, increasing Na^+ ions. Poor wash out of H^+ increases its concentration and causes the inhibition of mitochondrial oxidation of $NADH_2$. The increase in Na^+ causes the influx of Ca^{2+} which degrades protein and phospholipids[62]. Ischemic injury worsens the coronary damage and leaves endothelium more vulnerable. Neutrophils/platelets aggregating in the lumen of the coronary artery hamper the adequate blood flow[62]. These neutrophils have a role to play in inflammation. On reperfusion, H^+ ion outside the cell is

abruptly reduced to normal levels because it is washed out. This results in an increase in Ca^{2+} due to enhance Na^{+}-H^{+} and Na^{+}-Ca^{+} exchange[5]. Reperfusion also leads to formation of the free radicals due to abundance of the oxygen. Ca^{2+} and free radicals injury myocardium further during reperfusion. Moreover the vascular damage is maximum at reperfusion. The cells undergo necrosis/apoptosis during the reperfusion and ultimately resulting in the irreversible death of the myocytes because of reperfusion injury.

These all major mechanisms of myocardial reperfusion injury and the factors affecting these mechanisms are discussed below.

2.1.1 ATP Depletion and its effect on pH:

The energy metabolism shifts from aerobic or mitochondrial metabolism to anaerobic glycolysis after only 8 seconds of reduced arterial flow in ischemia[57,58]. This shift occurs as soon as the O_2 trapped in the tissue as oxyhemoglobin and oxymyoglobin is consumed. As the demand of the myocytes for energy far exceeds the supply from anaerobic glycolysis and from reserves of high energy phosphate (HEP), tissue ATP decreases and ADP begins to accumulate. Creatine phosphate, a major reserve source of HEP, decreases very quickly. It is 90% exhausted after 30 seconds of ischemia, whereas ATP declines more slowly. Late in the reversible phase of ischemia, however, 75% to 80% of the ATP present at the onset of ischemia has disappeared. Anaerobic glycolysis provides 80% of the new HEP produced in zones of severe or total ischemia[63]. Because little glucose is trapped in the extracellular fluid, anaerobic glycolysis utilizes glucose-1-P from glycogenolysis as its substrate. In this process, 3 μmol HEP is generated per each μmol glucose-1-P converted to 2 μmol lactate. The lactate and its associated H^{+} accumulate. As a direct result of the physiological events initiated during ischemia, acidosis develops as a result of inhibition of mitochondrial oxidation of NADH, inhibition of the vacuolar proton ATPase, increase in tissue CO_2 levels, excess glycogen turnover, anaerobic glycolysis, and ATP hydrolysis. After only 10 minutes of ischemia, the intracellular pH decreases to 5.8 to 6.0[65], and the load of intracellular osmotically active particles, lactate, inorganic phosphate, creatine, etc, increases markedly[64]. This osmotic load, however, causes only a modest increase in intracellular H_2O, because relatively little H_2O is available in the extra-cellular space of severely ischemic tissue to support the swelling process. This edema related to an increase in the sarcoplasmic space[63]. Tissue glycogen decreases and products of anaerobic glycolysis, such as glucose-1-P, glucose-6-phosphate, α-glycerolphosphate, and lactate, increase[58,66]. The adenine nucleotide pool is degraded as the ADP formed from the action of ATPases accumulates, because ADP is

being formed quickly, whereas rephosphorylation of ADP to ATP via anaerobic glycolysis is slowed by acidosis and lactate[67]. The HEP of ADP is captured for use via the action of adenylate kinase. In the process, AMP is formed and accumulates intracellularly, where it is degraded to adenosine. The adenosine diffuses to the extra-cellular fluid and is lost from the adenine nucleotide pool. In the extracellular fluid, adenosine is further degraded to inosine and hypoxanthine. Both metabolites accumulate. The result of these reactions is a reduction in the size of the adenine nucleotide pool (ΣAd), ie, Σ (ATP+ADP+AMP). Late in the reversible phase of ischemia, it falls to 30% to 40% of the initial level[68]. By creating acidic conditions, the cell primes itself for apoptotic cell death.

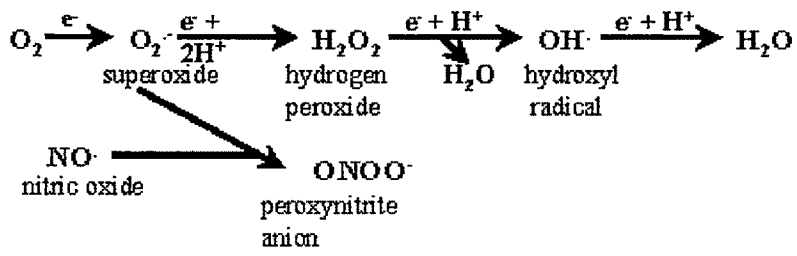
2.1.2 Free radical generation in myocardial reperfusion injury:

The myocardial damage is accentuated by the generation free radicals from different sources during the process of reperfusion injury. Here, we will first discuss, what are free radicals and their generation.

A free radical can be termed as any chemical species that contains one or more unpaired electrons in their outer orbit. Unpaired electron makes the molecule unstable and highly reactive; hence it quickly pairs with an electron from the surrounding molecule. This oxidation has two effects in the target molecule, first it is chemically modified and second, it will also become reactive, as it has unpaired electron. This will lead to oxidation of another surrounding molecule and a chain reaction will be initiated regenerating free radicals, thus destroying large number of cell components[69]. The free radicals are generated in three ways, a. hemolytic cleavage of covalent bond, b. loss of electron form a stable molecule and, c. an addition of electron to a stable molecule. This generation of the free radical depends on the stimuli exerted to the cell. The free radicals are of oxygen origin i.e. reactive oxygen species (ROS) and others are generated by nitrogen termed as reactive nitrogen species (RNS)[70]. ROS can be divided as radicals and non radicals. Radicals are Superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) and alkoxyl ($RO^{\cdot}$) comes in this category. The non radicals are hydrogen peroxide(H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). The nitrogen species are nitic oxide ($NO^{\cdot}$) and nitrogen dioxide ($^{\cdot}NO_2$). The non radicals in this category are nitrous oxide (HNO_2), peroxynitrite (O=NOO) and alkyl peroxynitrates (ROONO).

The burst of ROS seen during reperfusion may originate from a different cellular source than during ischemia and is not yet fully identified. ROS and cellular redox conditions regulate a large number of vital pathways (energy metabolism, survival/stress responses, apoptosis, inflammatory response, oxygen sensing, etc). While cellular systems may demonstrate reperfusion injury, there are few contradictions about the exact role of free radicals in the injury.

Under normal conditions, oxygen is reduced to H_2O in the myocardium via two paths. Mitochondrial electron transport reduces 95% of O_2 by tetravalent reduction to H_2O without any free radical intermediates. However, the remaining 5% of oxygen is reduced via the univalent pathway in which free radicals are produced. When oxygen accepts an electron the superoxide ($O2^-$) anion is formed. Superoxide is in equilibrium with its protonated form, $^{\cdot}HO_2$. During acidosis, as occurs in ischemia, the protonated form $^{\cdot}HO_2$ is favored and more reactive. $^{\cdot}HO_2$ is capable of oxidative injury to fatty acids and cell membranes, which is prevented within the cell by dismutation (primarily by superoxide dismutase) to H_2O_2. H_2O_2 is only directly toxic at high concentrations not thought to occur under normal conditions in cells. The cells have the endogenopus mechanism to neutralize this formed H_2O_2 by catalase and glutathione system. Superoxide formed as a byproduct of respiratory chain is safely neutralized to water. However, in ischemia/reperfusion condition this delicate balance is destabilized. As the ischemia intensifies, the defensive mechanism of the cells depletes and H_2O_2 become more empowered to generate the destructive hydroxyl radical ($^{\cdot}OH$)[71]. Metal ions, particularly iron, may play a role in generating hydroxyl radicals, hence the rationale for metal chelation during oxidative stress[71,72]. Hydroxyl radicals are extremely reactive and may cause direct cell membrane damage, lipid peroxidation, and damage to proteins and sulfhydryl bonds[73].



Iron and copper play a major role in the generation of free radical injury and facilitation of lipid peroxidation. Transition metal ions participate in the Haber-Weiss reaction that generates $^{\cdot}OH$ from $O_2^{\cdot -}$ and H_2O_2

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + HO^{\cdot} + HO^-$$

The Haber-Weiss reaction accelerates the non-enzymatic oxidation of molecules such as epinephrine and glutathione that generates $O_2^{\cdot -}$, H_2O_2 and subsequently $^{\cdot}OH$[73].

Reperfusion injury activates production of excessive quantities of reactive oxygen species. Molecular oxygen, when reintroduced into a previously ischemic myocardium, undergoes a sequential reduction leading to the formation of oxygen free radicals. Numerous potent oxidant radicals such as superoxide anion, hydroxyl radical, and peroxynitrite, are produced within the first few minutes of reflow and play a crucial role in the development of reperfusion injury[74]. Oxygen free radicals also can be generated from sources other than reduction of molecular oxygen. Enzymes, such as xanthine oxidase, cytochrome oxidase, and cyclooxygenase, and the oxidation of catecholamines have the major impact on the generation of ROS.

Reperfusion is also a potent stimulus for neutrophil activation and accumulation[75], which in turn serve as potent stimuli for reactive oxygen species production. Oxygen-derived free radicals produce damage by reacting with poly-unsaturated fatty acids, resulting in the formation of lipid peroxides and hydroperoxides that damage the sarcolemma and impair the function of membrane bound enzyme systems. Free radicals stimulate the endothelial release of platelet activating factor, which attracts more neutrophils and amplifies the production of oxidant radicals and the degree of reperfusion injury. Reactive oxygen species also quench nitric oxide, exaggerating endothelial injury and microvascular dysfunction. In addition to an increased production, there is also a relative deficiency in endogenous oxidant scavenging enzymes, which further exaggerates free radical mediated cardiac dysfunction.

2.1.2.1 Various enzymes and mediators assisting ROS generation:

Aldose reductase:

Aldose reductase (AR) is a member of the aldo-keto reductase family with remarkably broad substrate specificity. Pharmacological inhibition of AR protects perfused rat hearts from ischemic injury. Hwang et al, (2004)[76] demonstrate that hearts from human AR-overexpressing mice exhibit increased ischemic injury and poor functional recovery upon reperfusion. Increased AR activity is detrimental under ischemic conditions and impedes functional and metabolic recovery during reperfusion[76]. A potential mechanism by which AR influences ischemic injury involves energy metabolism and associated events. Ischemia results in an increased flux via the AR pathway. Increased flux via this pathway leads to utilization of NADPH by AR and NAD^+ by sorbitol dehydrogenase. This flux contributes to increases in the cytosolic NADH/NAD^+ ratio. Lactate/pyruvate ratio, a measure of the cytosolic NADH/NAD^+ ratio, was significantly higher in ischemic hearts than the normal myocardium. Sorbitol dehydrogenase in this pathway competes with glycolytic enzyme GAPDH for cytosolic NAD^+, increasing NADH/NAD^+ ratio and impairing glycolysis and glucose use. Studies have shown that AR inhibition or sorbitol dehydrogenase inhibition normalizes glucose metabolism in hearts[76,77].

Improving glucose use is critical for rescuing ischemic myocardium[78,79]. Myocardium is primarily dependent on glycolysis to meet its energy demands during ischemia. Blocking of glycolytic flux due to competition between GAPDH and sorbitol dehydrogenase for NAD^+ limits ATP generation and lowers the cytosolic NADH/NAD^+ ratio under ischemic conditions[80-83]. Ischemia ATP levels were significantly lowers and the cytosolic NADH/NAD^+ ratio was higher in ARTg (AR overexpressing) mice hearts compared with wild type. AR inhibition improved ATP levels and lowered the NADH/NAD^+ ratio in ARTg mice during ischemia. Thus decreased ATP levels in ARTg mice are due, in part, to impaired glycolysis.

NADH/NADPH oxidase enzyme:

Phagocytic cells generally contain the enzyme called NADPH oxidase. This enzyme prevents the infectious attack by producing ROS. Recently, it has been demonstrated that vascular NADH/NADPH oxidase significantly contributes to superoxide production in all components of the vasculature, i.e., the endothelium, the

medial smooth muscle, and the adventitia. Angiotensin II (AngII) which plays vital role in cardiac disease may activate superoxide production in vascular tissue via this enzyme. This enhanced production of superoxide has been implicated in the pathogenesis of AngII-induced vascular hypertrophy and endothelial cell dysfunction [86,87].

Xanthine oxidase enzyme and ROS:

Xanthine oxidase enzyme is closely linked to the ROS generation in ischemic conditions. Under normal physiological conditions, XO is a key enzyme in the purine degradation pathway. The enzyme generates the final product, uric acid (excreted by urine), and the byproduct, superoxide anion. Excess ROS production, during chronic hypoxia or in the presence of increased inflammatory cytokines, can enhance XO activity and its release into the plasma.

COX/iNOS on reperfusion injury:

The increase in the inducible form of nitric oxide synthase (iNOS) activation by the infarcted heart coincides with the appearance of infiltrating macrophages located at the border between the area of risk and the area of necrosis[88]. The iNOS has been proposed to interact with ryanodine receptors, thereby increasing calcium release from the sarcoplasmic reticulum[89]. NO also rapidly interacts with superoxide anions to produce the potent oxidant peroxynitrite ($ONOO^-$.). The formation of free oxygen radicals is increased during reperfusion, depending on duration and severity of the preceding ischemia; thus with more severe and prolonged ischemia free radical formation during the subsequent reperfusion is increased[90], subsequently also increasing the formation of peroxynitrite.

Myocardial production of prostacyclin (PGI_2) and thromboxane TXA_2 also increases[91]. The close relationship in time between activation of iNOS and production of PGI_2 and TXA_2 suggests an interaction between iNOS, the enzyme responsible for the production of NO, and cyclooxygenases (COX) activation[91-93]. COX isoenzymes produce prostanoids from arachidonic acid. Protanoids acts as vasodilator and reduces infarct size by inhibiting the platelet aggregation. COX exists in two isoforms; COX-1 is constitutively expressed, while COX-2 is induced primarily about by inflammatory cytokines[94]. Cytokines are reported to assist in ROS production. TXA_2 promotes platelet aggregation, initiates ventricular arrhythmias and increases infarct size[91].

Cytokines as ROS generators:

Recent studies suggest that cytokines not only induce reactive oxygen species (ROS) production but also are themselves induced by ROS[95]. In this regard, H2O2 can directly induce myocardial TNF production via the p38 MAPK pathway and, in turn, mediate myocardial dysfunction and apoptosis[95]. Neutrophil infiltrated mediated induction of NF-κB and AP-1 are linked to the generation of intermediate and non-lethal levels of reactive oxygen radicals, because the latter are present in large quantities during reperfusion[96].

2.1.3 Fatty acid metabolism in the ischemia/ reperfusion injury:

Circulating fatty acid levels increase after an acute myocardial infarction, such that during and after ischemia the heart muscle can be exposed to very high concentrations of fatty acids[97]. The detrimental effects of high plasma fatty acid levels on mechanical and electrophysiologic characteristics of the heart after ischemia reperfusion has been recognized for 20 years[97,98]. It also increases the severity of ischemic damage and has also been linked to a depression of mechanical function during aerobic reperfusion of previously ischemic hearts[99,100]. During reperfusion of the heart after ischemia, fatty acid oxidation can quickly recover and dominate as the source of ATP production. These high rates of fatty acid oxidation contribute to a marked decrease in cardiac efficiency during reperfusion[100]. However, if glucose oxidation is stimulated during reperfusion, a significant increase in cardiac efficiency results, with a parallel improvement in cardiac function[99]. One way to manipulate energy metabolism in reperfusion injury is to increase glucose metabolism and decrease fatty acid metabolism in the heart by decreasing circulating fatty acid levels.

2.1.4 Calcium overload and Na/H^+ pump:

During ischemia and early reperfusion, the accumulating intracellular protons stimulate the Na^+ /H^+ exchanger, increasing intracellular Na^+ levels. These sodium ions are eliminated via the Na^+ /Ca^{2+} exchanger, resulting in elevated intracellular calcium[101]. Stimulation of the β-adrenergic receptors during ischemia/reperfusion has also been reported to instigate calcium influx[102]. Calcium homeostasis is critical in normal cell functioning; mitochondrial uptake of calcium both transduces intracellular signals and aids the functioning of calcium sensitive matrix dehydrogenases[103]. Elevated calcium plays an important role in ischemia/reperfusion injury, through activation of a host of calcium-dependent enzymes including protein kinases, phosphatases, phospholipases,

proteases such as calpain, and endonucleases. This effect is due to calcium entry through L-type calcium channels or may be secondary to alterations in sarcoplasmic reticulum calcium cycling. Increased intracellular calcium can directly impinge on mitochondrial functioning. Elevated levels of intracellular calcium lead to excessive mitochondrial calcium influx characterized by mitochondrial swelling and intra-mitochondrial calcium deposits[105]. Calcium overload in isolated heart mitochondria has been demonstrated to induce cytochrome-*c* (inflammation) release. Furthermore, high mitochondrial calcium levels induce permeability transition pore (PTP) opening, allowing the release of calcium, cytochrome-*c*, NAD and apoptogenic factors into the cytosol[106,107]. Intracellular calcium levels can directly regulate the activity of calcineurin, endonucleases and proteases. DNase-I is calcium and magnesium-dependent endonuclease that cleaves DNA into oligonucleosomal fragments to give rise to the classical DNA ladder that is a hallmark of apoptosis[108].

2.1.5 Endothelial Dysfunction in reperfusion Injury:

Reperfusion injury leads to marked endothelial cell dysfunction[109]. Endothelium-dependent vasodilatation is impaired, whereas the responses to endothelium-dependent vasoconstrictors are exaggerated. Endothelin-1 and oxygen free radical, which act as potent vasoconstrictors increases after reperfusion and in-turn amplify coronary vasoconstriction and reduces blood flow. Furthermore, endothelial dysfunction facilitates the expression of a prothrombotic factors characterized by platelet and neutrophil activation which are important mediators of reperfusion injury. Once neutrophils make contact with the dysfunctional endothelium, they are activated, and in a series of well-defined steps (rolling, firm adherence, and transmigration) they migrate into areas of tissue injury through endothelial cell junctions[75,109].

2.1.6 K_{ATP} channel modulation in reperfusion injury:

The sarcolemmal membrane as well as the mitochondrial membrane of cardiomyocytes channels exhibits high density of ATP-sensitive potassium (K_{ATP}). The K_{ATP} channel is a weakly inward-rectifying K^+ channel that is inhibited by intracellular ATP and activated by intracellular nucleoside diphosphates. Both sarcolemmal and the more nebulous mitochondrial K_{ATP} channels in the cardiovascular system might have a physiological role in modulating cardiac function, particularly under conditions of metabolic stress, such as hypoxia, ischemia, and metabolic inhibition when intracellular

ATP stores are reduced. Under normoxic conditions, the K_{ATP} channel exists mainly in a closed, inactive form. Most likely, reperfusion provides oxygen to reactivate mitochondrial respiration but also causes a large production of oxygen free radicals (as the electron transport chain is in a reduced state) and a large influx of Ca^{2+} in the cytosol as a result of sarcolemmal damage. Mitochondrial Ca^{2+} transport is, therefore, stimulated at maximal rates. Mitochondrial Ca^{2+} accumulation causes profound alterations in the permeability of the inner membrane to solutes, leading to severe mitochondrial swelling. In addition, Ca^{2+} transport takes precedence over ATP synthesis and hence energy production.

However hypoxia during myocardial ischemia leads to fall in intracellular ATP concentration and ischemic metabolites (ADP, lactate, H^+) accumulate, the probability of the channel being open increases. This results in an enhanced outward repolarizing flow of K^+ and cell membrane hyperpolarization. Consequently, the myocardial action potential duration is shortened, the voltage-dependent calcium current and myocardial contractility are decreased thereby leading to ATP preservation during ischemia. Thus it is likely that mitochondrial-K_{ATP} activation may act to inhibit ATP waste. Hence, K_{ATP} channels may exert a protective property in myocardial ischemic diseases[110]. Recent studies reported that mitochondrial K_{ATP} channel opening rather than sarcolemmal (surface) K_{ATP} channel opening may play a dominant role in affording cardioprotection in ischemic hearts due to its energy-modulating property[100]. The non-uniform shortening of the action potential during myocardial ischemia by K_{ATP} channel opening may cause increased electrical inhomogeneity leading to development of arrhythmias during acute myocardial ischemia. Hence, K_{ATP} channel openers have been reported to be proarrhythmic associated with a tendency to increase infarct size during acute ischemia in isolated, perfused heart preparations in many studies[111,112]. However, few studies hinted at the protective effect of the K_{ATP} channel openers[113,114].

2.1.7 Reperfusion/reoxygenation induced contractures:

When hearts are reperfused after prolonged ischemia and or unsatisfactory intra-operative cardioplegia, reperfusion occasionally provokes the "stone heart" phenomenon, i.e. a stiff and pale heart resulting from massive muscle contracture and the concomitant loss of cellular protein content. This stone heart phenomenon belongs to the early phase of reperfusion, as it develops within minutes. Histologically, stone hearts

present hypercontracted myofibrils and ruptured cellular membranes. A similar pattern of contracture and necrotic cell injury, termed "contraction band necrosis", is also observed after regional ischemia and reperfusion in myocardium[115]. The extent of contraction band necrosis correlates well with the magnitude of macroscopic myocardial shrinkage during the first minutes of reperfusion, and with the magnitude of enzyme release occurring during the initial minutes of reflow[116]. It is the development of contracture that triggers reperfusion-induced necrosis. In ischemic myocardium, contracture develops by a rigor-type mechanism. A force, generated by slow cross-bridge cycling, is initiated when cytosolic ATP is reduced to a low (< 100AM) but non-zero level[117,118]. However, this is a temporary event, once the ATP stores are exhausted, the myocardial shortening stays fixed as all the cross-bridges between actin and myosin remain in an attached position. These contractions are moderate but it leads to cytoskeleton damage. The fragile cardiomyocytes, which are highly susceptible to mechanical damage, may recover after the restoration of energy supply by reperfusion[119].

The hyper-contraction induced by delayed reperfusion is more intense and cause more shortening of myofibrils as compared to ischemia rigor contractions. There are two independent causes of reperfusion-induced hypercontracture: Ca^{2+} over-load- induced contracture and rigor-type contracture.

Calcium overload-induced contracture is elicited in a cardiomyocyte if it develops Ca^{2+} overload during ischemia and is then rapidly re-energized. Ischemic cardiac cells become energy depleted and subsequently develop a Ca^{2+} overload of the cytosol due to an initial accumulation of Na^{+} and a subsequent uptake of Ca^{2+} through a reverse-mode operation of the sarcolemmal Na^{+}/Ca^{2+} exchanger. This Ca^{2+} calcium loaded cardiomyocyte at the end of ischemia, gets reoxygenated due to reperfusion. Unless the ATP synthesizing ability of mitochondria is not critically impaired, reoxygenation leads to rapid recovery of oxidative energy production. Resynthesis of ATP can enable cardiomyocytes to recover from the loss of cytosolic cation balance but it also reactivates the contractile machinery. Contractile activation is normally faster than Ca^{2+} recovery and this leads to an uncontrolled Ca^{2+} dependent contraction. The frequency of these Ca^{2+} peaks is influenced by an ongoing Ca^{2+} influx across the sarcolemma during the early phase of reoxygenation[119]. Part of the protective effects of cGMP-mediated effectors (NO, ANP) or cytosolic acidosis can also be attributed to contractile inhibition, as these agents reduce the Ca^{2+} sensitivity of myofibrils. Second, reducing SR-dependent Ca^{2+} oscillations can re-duce contracture.

The reoxygenation induced rigor contractures are less likely to occur after prolong ischemia. The low ATP content of myocytes during early phase of reperfusion provokes rigor contaction. The reoxygenated cells spend more time within the low cytosolic ATP phase that is more suitable to induce rigore contracture. In fact, the rigormechanism may become the major contributor to reoxygenation induced hypercontracture[120].The rigor contracture can be reduced at the time of reperfusion by improving the conditions for energy recovery, probably with application of mitochondrial energy substrates, e.g. succinate, with the aim of accelerating oxidative energy production. The gap junction-mediated communication between ischemic cells allows spreading of cell injury during myocardial reperfusion[121]. The passage of sodium through gap junctions from hypercontracting cells to adjacent cells and the subsequent change in cytosolic Ca^{2+} levels through reverse-mode Na^{+}/Ca^{2+} exchange may result in propagation of contracture[122].

2.1.8: Inflammation in reperfusion injury:

Neutrophils exerts its response to reperfusion only when it is activated and it is activated by vast array mediators generated from multiple cell types in myocardium like endothelium, mast cell and myocytes. Complement C5a stimulates neutrophil adherence to vascular endothelium and associated decreases in blood flow. TNFa stimulates neutrophil superoxide anion generation and adhesion[123-125]. The levels of complement components and cytokines are elevated during reperfusion injury. Cytokines such as TNF-α can be released by degranulation of mast cells during ischemia/reperfusion[126-127]. Other factors released by mast cells such as histamine, serotonin[128] and PAF[129] participate in the inflammatory response to ischemia/reperfusion directly or by stimulating the release of other cytokines such as IL-6. Cytokine generation in myocardium is regulated at the transcriptional level by NF-κB, which is activated by cytokines themselves or by ROS[130,131], which itself induces the myocardial damage in reperfusion injury. Lipid mediators act as triggers of neutrophil activation and adherence. The arachidonic acid metabolite leukotriene-B4 (LTB_4) is released into ischemic/reperfused myocardium primarily by activated neutrophils. LTB_4 is a potent chemoattractant factor for neutrophils.

Without reperfusion, neutrophil infiltration is restricted to primarily the border zone of the area at risk myocardium, and few neutrophils are found toward the center of the necrotic zone[132]. However, neutrophil infiltration and accumulation is accelerated and increased with reperfusion after reversible occlusion, with a greater accumulation in the

subendocardium compared to the sub-epicardial myocardium[134]. The neutrophil mediated injury had specific consequences on the myocardium. Neutrophil activation results in necrosis by various mechanisms, like ROS generation, release of enzymes, stimulation of cytokines (IL-8) which causes the vasoconstriction. Neutrophils promote the interaction with platelets that may potentiats ischemia/reperfusion injury. It also extends the infarct size and the degree of apoptosis during later phases of reperfusion[133].

The coronary vascular endothelium is a single cell layer front line defense against inflammatory reactions. The endothelium is not simply an inert barrier[135] but is an active participant in preventing neutrophil adherence via nitric oxide[136,137] and adenosine[138] as well as in recruiting neutrophils. Neutrophils can directly cause damage to coronary vascular endothelium by adherence-dependent mechanisms involving the early adhesion molecule P-selectin[139]. Endothelial dysfunction was also observed in the early minutes after the start of reperfusion, and has been linked to progressive interaction with and adhesion of neutrophils[140]. Thus inflammation, through its mediators inflicts significant injury on myocardium during the reperfusion of the ischemic myocardium.

2.1.9 'Apoptosis' a programmed cell death during reperfusion injury:

Although a large portion of the cell loss during cardiac ischemia and reperfusion occurs through necrosis[141], there is presently increasing interest in the possibility that myocardial cell death may also occur through apoptosis[142]. Unlike necrosis, apoptosis proceeds through a genetically programmed series of biochemical and morphological steps designed to avoid the indiscriminate release of cytosolic contents and the ensuing inflammatory response[143]. The salient features of this type of cell death are chromatin condensation, endonuclease fragmentation of internucleosomal DNA to multiples of 180- to 200-bp fragments, and cell fragmentation into small membrane-bound vesicles. Since mammalian cardiac myocytes essentially rely on aerobic energy metabolism, it has been assumed that cardiocytes die in a catastrophic breakdown of cellular homeostasis (i.e. necrosis), if oxygen supply remains below a critical limit. Recent observations, however, indicate that a process of gene-directed cellular suicide (i.e. apoptosis) is activated in terminally differentiated cardiocytes of the adult mammalian heart by ischemia and reperfusion, and by cardiac overload as well. Apoptosis or programmed cell death is an actively regulated process of cellular self destruction, which requires energy and de novo gene expression, and which is directed by an inborn genetic program. The final result of this program is the fragmentation of nuclear DNA into typical 'nucleosomal ladders'.

A number of characteristic morphological changes are known to accompany apoptosis[144,145], their appearance in any given tissue may be dependent on the type or duration of injury. The fact that reperfusion accelerated the appearance of apoptotic nuclei in the reperfused myocardium suggests that reperfusion may actually increase the activity of the apoptotic mechanisms in cells committed to this form of cell death. The reason for this acceleration of apoptosis is not clear at the present time but may be attributable to the enhanced inflammatory response in reperfused myocardium, particularly the early sequestration of neutrophils. The causes of apoptosis in ischemic or reperfused myocardium remain obscure. Moreover, it is not yet clear whether myocardial apoptosis is triggered consistently during both ischemia and reperfusion. Few of the causes of apoptosis are discussed here.

ROS-induced cardiac apoptosis is mediated through several signalling systems, including intracellular Ca^{++}, cytokines, lipid oxidation, and proto-oncogene activation. The perturbation of intracellular Ca^{++} homeostasis by the cellular redox state can aggravate free radical reactions and can activate endonucleases via activation of caspases, key enzymes in the apoptotic pathway. Additionally, many recent studies have demonstrated that the intrinsic degree of oxidant production regulates cellular susceptibility to apoptosis through both p53-dependent and p53-independent pathways. Different ROS activate distinct signalling pathways for programmed cell death in cardiac myocytes[146].

A key phenomenon of apoptotic cell death is the activation of a unique class of aspartate-specific proteases, these proteases are collectively termed as caspases[147]. Target proteins for caspases comprise a plethora of different proteins, including nuclear proteins; proteins involved in signal transduction, and cytoskeletal targets[148,149]. Most of these protein substrates appear to be cleaved by caspases-3 and -7. Interneuclosomal DNA fragmentation requires the prior cleavage of a cytoplasmic inhibitor of the apoptosis-specific endonuclease (inhibitor of caspase-activated DNAse, DNA fragmentation factor) [150]. One of the best characterized pathways for the initiation of apoptosis involves the binding of extracellular death signal proteins (TNF-α, FasL, TRAIL TRAIL (related apoptosis-inducing ligand) and Apo-3L) to their cognate cell surface receptors[151,152]. Induction of apoptosis by FasL and TNF-a critically depends on caspase activation[153].

In addition to this, nuclear translocation of the transcription factor nuclear factor-kB (NFkB) and activation of c-Jun N-terminal kinase (JNK) are initiated and plays important role in apoptotic cell death[154]. Activation of the JNK pathway is implicated in

the initiation of apoptosis by different stress stimuli. In a recent report, even Fas-induced apoptosis was shown to be partly dependent on JNK activation, which was mediated by a novel signal transduction molecule termed Daxx. The proapoptotic effect of p53 has been linked to the p53-induced expression of Fas, bax, and IGF binding protein-3[155].

Since antioxidants reduced the extent of p53-induced apoptosis, oxidative stress seems to constitute an important intermediary step in p53-mediated apoptosis[156]. Survival factors like interleukin-3, nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), or platelet-derived growth factor protect cells from undergoing apoptotic.

Chromatin condensation and margination that result in a "half-moon" or "horseshoe" appearance of the nucleus are typical features of apoptotic cell death.The most commonly used techniques to detect apoptosis are based on the fact that during apoptosis the genomic DNA is cleaved within internucleosomal DNA segments by an endonuclease selectively activated during apoptosis[157].

2.2 Cerebral focal ischemia/reperfusion injury:

Few realms of disease are as devastating as neurological ones, because such damage can destroy our very essence of life. Stroke and following reperfusion injury is a major source of disability and thus much research is being placed on the treatment and prevention neurological well being. Various neuroprotective strategies include free radical scavengers, excitotoxicity inhibitors and calcium channel blockers. Numerous new intervention are being investigated for the prevention of the severe damage caused by the cerebral reperfusion injury. To list, few of these are, various hormones, tissue plasminogen activators and antibodies to adhesion molecules.

Pathogenetically, stroke integrates a heterogeneous group of diseases. Ischemic/reperfusion injury occurs in conditions like stoke. Vessel occlusions account for 85% of all strokes, while primary intracerebral bleeding has a relatively low incidence of approximately 15%. Embolisms, of either arterio-arterial or cardiac genesis account for 75% of all cerebral vessel occlusions and are the most frequent cause for focally impeded blood flow within the brain. Microangiopathical causes, namely in-situ-thromboses and hyalinoses of the arterioles, occur in about 20% of all cases. Hemodynamic infarctions as a result of high-grade stenoses of the cerebral arteries are comparatively rare (less than 5%)[158,159]. The reperfusion of ischemic brain brought by various interventions leads to

cellular injury. The animal model of focal cerebral ischemia is induced, which clinically corresponds to ischemic stroke.

Brain damage in stroke is the result of a plethora of highly complex mechanisms leading to infarct maturation. As the brain has a very high demand for oxygen and glucose, a disruption of the circulation in the areas affected by vascular occlusion leads to a depletion of substrates within minutes. Furthermore, potentially toxic metabolites accumulate. This leads to an energy deficit within the affected cells. The result is functional or even structural damage to the cells, depending on the degree and duration of the energy deficit.

The degree to which blood flow is reduced directly determines the changes in three variables that are almost undoubtedly the genesis of all future changes. Two of these, inhibition of electron transport and decreased ATP, appear to be directly related to the extent of flow reduction. The third variable, free radical production, is likely to be a more complex function of flow rate because it is activated both by electron transport inhibition and also by oxygen so that it will be maximal at blood flow values greater than zero. Ischemic cell death is initiated by changes that result directly from inhibition of oxidative phosphorylation and create an early maelstrom of activity. These changes include decreased pH, decreased ATP, initiation of free radical production by the mitochondrial chain, increased cell Na^+ and membrane depolarization as a result of the loss of ATP substrate for the Na^+-K^+ pump. These lead to secondary changes in ion and chemical concentrations which, in concert with the initiating changes, result in activation of damaging processes. Thus the process of cell death has at least three major stages: an early development of ionic and chemical changes, a resulting activation of cascading events, and a subsequent change in critical functions and structures that denote or lead to cell death by one of three different routes[160].

Almost all focal ischemic models, whether larger mammal primarily involve occlusion of one middle cerebral artery[161] widely used method. Flow following temporary ischemia is somewhat compromised by the partial occlusion of the carotid arteries with the filament[162]. Also, even a temporary there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, and hence there are different metabolic conditions within the affected site. It is an invaluable model for stroke and is thus widely studied. A newer and now widely used approach to proximal MCA occlusion is the insertion of a nylon suture into the carotid artery past the point at which the MCA branches so that the latter is occluded at its origin[162-164]. Used optimally, the coefficient of

variation in lesion size is 8% and of blood flow is 35%[162]. A similarly small coefficient of variation in lesion size, along with a large lesion of 250 mm^3 at 24 h, was obtained with the silicone-coated suture in rats[165]. A temporary occlusion leads to a long-term increase in core temperature at ;1.5°C due to decreased circulation and damage to the hypothalamus, at least in Wistar rats. Finally, there is quite extensive damage to small arteries in the ischemic field, with loss of endothelium and muscle damage[166]. This occurs after normal insults (e.g., 6-h ischemia or 2-h ischemia and several hours of reperfusion). In proximal MCA occlusion, the core is in the lateral portion of the caudate putamen and overlying parietal/somatosensory cortex . Most other regions of neocortex and entorhinal cortex, as well as the medial caudate-putamen, constitute the penumbra[161]. The acute events triggered by the focal perfusion deficit in the brain and the chronic aspects of neuronal plasticity and regeneration are discussed in this section.

2.2.1 Ionic and metabolic changes in the core and penumbra after cerebral I/R injury:

The biochemical and ionic changes triggered by stroke strikingly different in core and penumbra. These differences in metabolism strongly suggest different mechanism for the cell death in these two regions. The core undergoes rapid anoxic depolarization within 1–3 min with a concomitant rise in extracellular K^+ to ;70 mM, which may show some short intermittent returns to baseline[167]. There is a large decrease inextracellular Ca^{2+} within 1–2 min at flow levels that are present in the core, signifying entry into the tissue. During the reperfusion period, after 2 h temporary focal ischemia, extracellular K^+ returns to control levels for 6 h before rising slightly (to 5 mM) for the remainder of a 24-h recirculation period. Despite this return to near normalcy, the insult produces severe damage with a large infarct including the entire core[167]. This massive disturbance of the ion homeostasis and the related passive influx of water lends to cell and tissue edema. These changes account for the osmotic lysis and the lytic cell death is also referred as necrosis, which is as a hallmark of the core of the infarct[168].

On the other hand in penumbra, there is no permanent anoxic depolarization with concomitant ion changes during the ischemia as collateral provide residual circulation. Presumably ATP levels, which are maintained at 50–70% of normal, do not fall enough to allow the anoxic depolarization. However, there are sporadic transient depolarizations[169] that are here termed "intraischemic depolarizations"[170]. They arise differently from

anoxic depolarizations. The depolarizations may actually originate from glutamate release in the infarct core.

2.2.2 Energy metabolism in stroke:

The ATP levels in core fall to 25% of basal values and remains there between 5 min and 4 h of ischemia[171]. The damage is severe even after restoring the ATP level up to two-third of the normal, for at least 4 h, whereas the ATP levels in penumbra average around 50-70 % of normal during ischemia. The glucose utilization fluctuates during the early times and 3.5 h of the ischemia. During ischemia, the blood flow in penumbra declines but it is still much higher than that of the core.

Levels of ATP during the reperfusion period after temporary ischemia do not seem to recover much from their ischemic values[172,173]. Glucose utilization in some penumbral tissue is reduced to well below normal during early reperfusion, and this is considered a reliable indicator of the tissue that will later become part of the infarction [162]. Presumably, the low glucose metabolism indicates severe cell damage of some kind at this early stage. Despite the rapid recovery of ion homeostasis recovery by reoxygenation, it is almost impossible to prevent damage by any intervention during the reperfusion period. It has also proven extremely difficult to prevent core damage by any single intervention, even before the onset of ischemia, when ischemia lasts for 2–3 h. Hence we have administered the drug individually or in combination before reperfusion to garner the maximum benefits of these interventions in stroke.

2.2.3 Free radical generation in cerebral reperfusion injury:

The increase in free radicals becomes larger and unambiguous during the early reperfusion period. In normal conditions, the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. Ischemia and reperfusion creates several conditions that could account for the increased net production of free radicals. As a consequence of ischemia and particularly of reperfusion, reactive free oxygen radicals such as superoxide, hydrogen peroxide, and hydroxyl radical are generated. The reaction product of superoxide and nitric oxide, which is generated after the activation of calcium–calmodulin-dependent types of nitric oxide synthase (NOS), is the highly reactive peroxy-nitrite radical. Radicals can react with virtually any cellular component (carbohydrates, amino acids, DNA, and phospholipids) and damage them[174].

Xanthine/Hypoxanthine Oxidation:

The breakdown of adenine nucleotides during ischemia leads to accumulation of hypoxanthine within 10 min, which is then metabolized by xanthine oxidase (XO) or xanthine dehydrogenate (XDH). Xanthine oxidase produces free radicals, but XDH does not. The basal level of XO in brain tissue is similar to that in other organs such as liver and intestine[175]. The reaction for generating superoxide is as follows

$$\text{Hypoxanthine} + O_2 \longrightarrow O_2\text{-}\cdot + H_2O_2 + \text{urate}$$

NO and peroxinitrate generation:

Nitric oxide appears to rise during focal ischemia, the transient increase in levels may go up to 2 μM. Nitric oxide is genereaed by neuronal or endothelial NOS in an oxygen-dependent reaction that is activated by Ca^{2+}/calmodulin in most neurons and endothelial cells[176]. The Ca^{2+}/calmodulin directly activates the enzyme and may also remove a phosphorylation-induced block by activating calcinurin[177]. Both neuronal and inducible NOS are upregulated in focal ischemia. Peroxynitrite is generated by the reaction between superoxide and NO

$$O_2\text{-}\cdot + NO\cdot \longrightarrow ONOO^-$$

Accumulation of Eicosanoids:

Oxidative metabolism of accumulated arachidonic acid[178] via the cyclooxygenase (COX) pathway[179] or via the lipoxygenase pathway[180] leads to superoxide or OH· production. Phospholipid breakdown into arachidonic acid is then further activated by free radicals[181]. There is a manifold increase of FFA, including arachidonic acid, during focal ischemia[182]. These two enzymes (NOS and COX) that are intimately related to free radical production are upregulated during the 6- to 24-h period after temporary focal ischemia, as they are after global ischemia. Both iNOS[176] and COX-2 are upregulated in the penumbral region during this period. The former is in endothelial cells and invading neutrophils[176], whereas the latter is in neurons. Cyclooxygenase-2 (prostaglandin H synthase) generates superoxide radicals. The up-regulation of these enzymes might lead to NO and superoxide synthesis during the later phases of reperfusion. Although they are in different

cell types, they might well react to form peroxynitrite because both NO and superoxide can diffuse for several hundred microns[183].

Altered mitochondrial function:

Free radicals are generated at brisk pace by the organelles in mitochondria. When electron carried becomes highly reduced, as much as 2% of the electron flow leads to direct singlet-electron reduction of oxygen and formation of superoxide[184]. These reactions very prominently occur in focal ischemia. Accumulation of Ca^{2+} and opening of mitochondrial permeability transition pore (MPT) at some stage of ischemia assist in generation of free radicals.

Role of Monoamine accumulation:

H_2O_2 is generated from accumulated catacholamines via monoamine oxidase (MAO) in the 5 min after ischemia. Superoxide and NO are usually the first free radicals formed, but superoxide is not very reactive. The formation of $ONOO^-$ is likely to mediate much of its toxicity. In addition, though, the reaction products of superoxide, $OH^\cdot$ or $HO_2^\cdot$-are very reactive and more likely to mediate toxic effects[149]. Formation of $OH^\cdot$ is strongly favored by the decreased pH that prevails during and after ischemia[185,186] and also by free iron[149], as shown below in the Fenton reaction

$$O_2^- + 2H_2O \longrightarrow + H_2O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\cdot$$

2.2.4 pH changes during stroke:

Intracellular pH(pHi) fslls by 0.5-1 unit during cerebral ischemia. At the core, where blood flow was reduced to 25% at 10 min and to 10% by 3 and 4 h, pHi fell to 6.6, 6.2, and 6.1 at those times. In the penumbra, where flow was reduced to 40%, there was no significant pHi drop at 3 h and a slight drop (to pH 6.75) at 4 h[187]. This acidity exacerbates free radical damage; the pH profile during reperfusion is a very important parameter. However, decrease in pH alone cannot exert the extensive damage. The early decrease in pHi probably results from the immediate blockade of oxidative phosphorylation and continued flux through the glycolytic flux[188]. The other mechanism is assumed to be the activation of glycolysis stemming from the lowered ATP. Fall in pHi correlates with increased lactate concentration as blood glucose increased[189]. The evidences showed that the reduction in pHi during focal ischemia makes a major

contribution to development of the damage. This so-called "lactate–acidosis-hypothesis" is frequently cited in order to explain the glucose paradox of cerebral ischemia. The "paradox" is that there is experimental evidence that high supply of glucose, the most important source of energy of the brain, in cerebral ischemia does not reduce tissue damage as one should think, but instead increases it. But this view is not unchallenged[185].

2.2.5 Anoxic depolarisation and Na^+-K^+ pump in cerebral reperfusion injury:

Anoxic depolarization is the cardinal feature of anoxia/ischemia *in-vivo*. This anoxic deploarisation is observed especially in core region and not in the penumbra[190]. The phenomenon is characterized by a large (10-30 mV) extracellular negative direct-current shift in cell bodies of the ischemic tissue. This is often accompanied by precipitous decrease in extracellualr Na^+, Cl^- and Ca^{2+}. Anoxic depolarization needs ATP for its initiation. The conditions like hypoglycemia where the rate of ATP depletion is increased in hippocampal slices, shortens the time to anoxic depolarization[191]. The average value of ATP is 40% at the time of anoxic depolarization and the depolarization occurs within 5 sec of the time at which ATP suffered a precipitous fall (60-90 Sec)[189]. This anoxic depolarization is due Na^+-K^+ pump inhibition as it is essentially independent of extracellular Ca^{+}[193], NMDA receptor activation and only very slightly on non-NMDA glutamate receptors. The ability of pump to cause rapid depolarization is thought to be due the very high surface to volume of neuronal elements, which means that pump inhibiton produces rapid changes in ion concentrations[192]. If pump inhibition is indeed the basis for the anoxic depolarization, the shift in K^+ as well as any specific permeability changes is likely to be responsible for the majority of the depolarization[194]. The neuronal damage is strongly correlated with depolarization.

Furthermore, there are massive biochemical changes and ionic changes at the time of the anoxic depolarization, including the very large increase in FFA during ischemia[189]. Hence, anoxic depolarization contributes to damage as it is dependent on the decrease in ATP levels, and increase in Na^+, two factors which almost undoubtedly strongly activate the damaging events.

2.2.6 Inflammation in reperfusion injury:

The early stage of inflammation, which starts a few hours after the onset of ischemia, is characterized by the expression of adhesion molecules at the vascular endothelium as well as by circulating leukocytes. Thus, leukocytes adhere to the

endothelium and transmigrate from the blood into the brain parenchyma. The interaction between the endothelium and circulating leukocytes is of decisive importance for stroke-induced brain inflammation. Neutrophil granulocytes accumulate in the cerebral microvessels of the penumbra, which leads to an additional disruption of microcirculation. Activated leukocytes (granulocytes, monocyte/macrophages, lymphocytes) as well as neurons and glial cells (astrocytes, microglia) produce cytokines and chemokine. In particular, proinflammatory cytokines such as TNF-α , IL-1, and IL-6 play an important role as mediators of the inflammatory phase of cerebral ischemia [195]. IL-1 β caused increase in the formation of nitrotyosine which acts as substrate for free radical generation and inflammation. Besides the expression of proinflammatory cytokines, anti-inflammatory cytokines such as TGF-β1 and IL-10 are also induced. As these cytokines downregulate inflammation, they have a protective effect in the context of cerebral ischemia[196]. In addition to the cytokines, there are two enzymes that are essential in the stage of inflammation: inducible NO-synthase (iNOS) and cyclooxygenase-2 (COX-2).

iNOS is implicated in the generation of cytotoxic peroxynitrite. COX-2 is mostly expressed in the penumbra. By means of the production of free oxygen radicals as well as prostanoids, COX2 is likely to have a damaging effect on the penumbral tissue[195].

2.2.7 Phospholipid metabolism:

Breakdown of phospholipids could, in principle, cause major changes in membrane function and also changes in signaling capabilities resulting from loss of phosphoinositides. Furthermore, the breakdowns lead to major increases in FFA production and hence, also, free radical formation. All these are potentially damaging. The oxidative metabolism of arachidonic acid, which is where free radicals are generated, will be greatest during reoxygenation. FFA and arachidonic acid in the CA1 region remained elevated for at least 1 day after 5 min of ischemia[197]. A prolonged generation of FFA in a vulnerable region, and this could be important in damage. Total FFA were increased 4- to 10-fold between the first 15 min and the end of 60 min of MCA occlusion[198]. If ischemia was reversed after 1 h, FFA returned toward baseline, and there was then a large secondary increase takes place at 16 and 24 h of reperfusion.

During cerebral ischemia, free fatty acid concentrations are markedly increased, the largest increase being that of arachidonic acid[199]. Ca^{2+}-activated phospholipases C and A_2 result in phospholipid hydrolysis, while resynthesis of phospholipids requires ATP. As

a result, ischemia-induced Ca^{2+} influx and energy failure promote free fatty acid release and concomitant membrane damage. Free fatty acid metabolism has multiple other adverse effects including inhibition of oxidative phosphorylation, oxidative conversion of free arachidonic acid *via* the cyclo-oxygenase pathway to eicosanoids (thromboxanes and prostaglandins), free radical generation and lipid peroxidation-mediated chain reactions, and cytotoxicity from lipid peroxidation products (e.g. 4- hydroxynonenal), which may stimulate apoptosis. It also activates platelet activating factor which is also responsible for explicit increase in Ca^{2+} in neurons. The membrane damage induced by this phospholipids break down is of importance in an organelle other than plasmelemma (eg. Mitochondria). Even the loss in phospholipids content is small in nonmitochondrial membrane may be small but the studies emphasizes the importance of membrane damage[200].

2.2.8 Blood brain barrier damage in cerebral reperfusion injury:

The integrity of the BBB depends on the interaction of the cellular matrix, which is composed of endothelial cells and astrocytes. Because of cerebral ischemia, this cellular matrix and the intercellular signal exchange is damaged. Proteases, especially matrix-metalloproteases (MMPs) play an important role at this. A matrix metalloproteinase, MMP-9, was upregulated in endothelial cells during the first 24 h of permanent ischemia, probably by cytokines[201]. There is a major extracellular edema that develops early during the focal ischemic insult, largely due to activation of Na^{+} transport across the blood-brain barrier[175]. Reperfusion and very prolonged permanent ischemia both cause much more dramatic changes. There is damage to the endothelial cell layer, along with extravasation of protein and protein-bound Evans blue, as early as 30 min after recirculation following 60-min ischemia. BBB is highly sensitive to temperature variation and this temperature sensitive nature of BBB is a probable cause of brain damage brought about by inflammatory reactivity. The increased permeability of BBB is responsible for the transfer of leukocyte recruitment mediators across barrier after hyperthermia during cerebral ischemic condition in rats[20]. Indeed, edema is often used as a measure of damage. Increased edema may simply reflect local blood-brain barrier damage caused by the damaged neurons, glia, and endothelial cells.

2.2.9 Apoptosis:

Apoptosis, a programmed cell death takes place in a similar time frame as inflammation. It commences hours after ischemia onset and lasts for days. This delayed cell death mostly occurs in the penumbral region and is thus temporally and spatially different from the fast neuronal and glial cell death (pan-necrosis, minutes to hours) in the core. Apoptosis is characterized by a biochemical cascade that leads to the activation of certain kinds of proteases: the caspases. Caspases catalyze the destruction of the cell, its compartments and molecules. The characteristic histological features of apoptosis are membrane-blebbing, chromatin condensation, shrunken cytoplasm with intact organelles, apoptotic bodies[185].

In apoptosis, there are discriminate intrinsic (i.e., basically mitochondrial) and extrinsic activation pathways. The extrinsic pathway is induced by activation of the FAS-receptor, e.g., through binding of TNF-α. Intrinsic activators of apoptosis in the context of ischemia are elevated intracellular levels of calcium, reactive oxygen species, glutamate, and DNA damage. By damaging the mitochondrial membranes, both pathways directly or indirectly lead to the activation of caspases. Caspases latently exist in every cell as inactive precursor proteins and are activated by the cleavage of the precursor protein. Caspases 1, 3, 8, and 9 are involved in cerebral ischemia. Caspases 8 and 9 are initiator cascades and stand at the beginning of the signaling cascade. Caspase 8 and 9 activate the execution caspases like caspase 3. Caspase 1 is involved in the activation of cytokines [203-205], and links inflammation with apoptosis. Caspase 3 has a central part in the apoptotic signaling cascade, not only in cerebral ischemia[205].

DNA-repair enzymes like poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinases (DNA-PK) are the substrates that are metabolized by caspase 3. PARP identifies single strand disruptions and mediates DNA-repair through the generation of ADP-ribose polymers. However, PARP reduces the energy pool of the cell because it consumes ATP. Although partially controversial, the use of ATP by PARP is considered cytotoxic[206]. The cleavage of the inhibitor of caspase-activated DNAse (ICAD) is of particular importance, as caspase-activated DNAse (CAD) is activated by this mechanism. CAD leads to the characteristic DNA-laddering[205]. Besides the destruction genetic information, structural proteins (e.g., laminin, actin, gelsolin) that are essential for the integrity of the nucleus and the cell are cleaved by caspases. Together, cytochrome c, the cytosolic protein Apaf-1, caspase 9 precursor protein, and ATP form the apoptosome. The apoptosome activates caspase 9 and leads to a sequential activation

of the downstream caspases. The re-lease of cytochrome c itself depends on Bcl-2 proteins. Cytochrome c is also released, if the mitochondrial membrane is directly damaged by free radicals[205].

The PI 3K/Akt-pathway, which is activated by neuro-protective factors, such as erythropoietin, plays an essential role in this neuroprotective pathway. Thus apoptosis is an intriguing process including wide array of mediators and mechanism which lead to programmed cell death in the tissue[168].

2.3 Markers of the ischemia/reperfusion injury:

Various mediators and their interrelated mechanisms accentuate the reperfusion injury. In the last few decades the need for early prognosis of the injury was considered as a vital prerequisite for the treatment and the management of the reperfusion injury. The research was focused on development of new markers of the injury for proper clinical approach to avoid the excessive damage and further complications. Few of the bio-indicators of the reperfusion in jury are discussed in this section.

2.3.1 Oxidative stress/ antioxidant biomarkers:

Oxidative stress caused by the production of free radicals is postulated as one of the earliest mechanisms for the damage to the tissue in the reperfusion injury. The generation various ROS can be assessed by the measurement of endogenous antioxidant enzymes and the level of reduced reduced glutathione. Lipid peroxidation during the injury is also of great importance to predict the damage caused by the oxidative stress.

2.3.1.1 Superoxide dismutase (SOD):

Under normal conditions, oxygen is reduced to H_2O in the cell via two paths. Mitochondrial electron transport reduces 95% of O_2 by tetravalent reduction to H_2O without any free radical intermediates[207]. However, the remaining 5% of oxygen is reduced via the univalent pathway in which free radicals are produced. When oxygen accepts an electron the superoxide (O_2^-) anion is formed. Superoxide is in equilibrium with its protonated form, $^{\cdot}HO_2$ .During acidosis, as occurs in ischemia; the protonated form $^{\cdot}HO_2$ is favored and more reactive. $^{\cdot}HO_2$ is capable of oxidative injury to fatty acids and cell membranes, which is prevented within the cell by dismutation (primarily by superoxide dismutase) to hydrogen peroxide (H_2O_2). H_2O_2 is only directly toxic at high

concentrations not thought to occur under normal conditions in cells. Cells have a well-adapted method to continue the reduction of H_2O_2 to H_2O via catalase or the glutathione system. In this manner superoxide, a byproduct of respiration is safely metabolized to water (Becker, 2004).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (k_{catalytic}\ 2\text{-}4 \times 10^9\ M^{-1}s^{-1})$$

The H_2O_2 formed here is metabolized to water that is shown in other section.

The generation of reactive oxygen species in combination with the ischemia-induces decrease in endogenous antioxidant (SOD) enzymes and leaves the tissue extremely vulnerable to the ischemic damage. Superoxide which generated here is a key constituent in oxidative stress. It is derived from various sources at different stages of reperfusion. There are three major endogenous superoxide dismutases. Cu,Zn-SOD (SOD1) is principally found in the cytosolic and lysosomal fractions, but is also in the mitochondrial intermembrane space[208]. MnSOD (SOD2) is found in the mitochondrial matrix MnSOD is induced by changes in the cellular redox state, inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon gamma (IFN-γ), lipopolysaccharide (LPS). MnSOD constitutes approximately 10-15% of the total SOD activity in most tissues. Overexpression of MnSOD protects the tissue from reperfusion injury induced damage. However, CuZnSOD is not required for normal development and survival in mice, the overexpression of CuZnSOD reduces the ischemic damage resulted form the ischemia/reperfusion injury[209]. Extracellular SOD (SOD3) is also expressed in brain but in substantially lower concentrations than SOD1 or SOD2[201]. EC-SOD, a tetrameric protein, is secreted into the extracellular compartment[211]. iNOS also has been shown to upregulate ECSOD after NF-κ B activation. EC-SOD is presumed to provide defense against superoxide present in the extracellular space (e.g. produced by membrane-bound NAD(P)H oxidase or secreted by inflammatory cells[212].

Thus from all these data, it is clear that SOD enzyme has a critical patholphysiological role in the oxidative stress induced damage and the mimetics of the SOD may have the clinical importance to restrict the injury.

2.3.1.2 Catalase (CAT)

SOD dismutates superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide has modest oxidative potential and can freely cross cell membranes. Through the iron-

catalyzed Haber–Weiss reaction (superoxide-driven Fenton chemistry), hydrogen peroxide can be converted to hydroxyl radical[213]. Elimination of hydrogen peroxide is therefore critical to the efficacy of SOD in reducing oxidative stress. Catalase and glutathione peroxidase (GPx) serve this purpose[210]. Catalase is mainly localized to peroxisomes. Catalase detoxifies H_2O_2 enzymatically by the following mechanism.

1. Catalytic

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$ * note oxygenates

2. Peroxidative first substrate is H_2O_2

$$ROOH + HQOH \longrightarrow QO + ROH + H_2O$$

A. $H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$

B. $ROOH + AH_2 \rightarrow H_2O + ROH + A$

Thus catalase metabolizes H_2O_2, prevents possible lipid peroxidation. NADPH has a vital role in CAT activity during the oxidative stress. NADPH assists CAT by preserving it from excessive H_2O_2 inactivation and provides a source of GPx during stress. Polyethylene glycol conjugated catalase and liposome-entrapped catalase protects against oxygen toxicity, but greater benefits are accomplished with the combination of PEG-SOD and PEG-CAT or liposome-entrapped SOD and CAT.

2.3.1.3 Glutathione depletion and role of glutathione peroxidases:

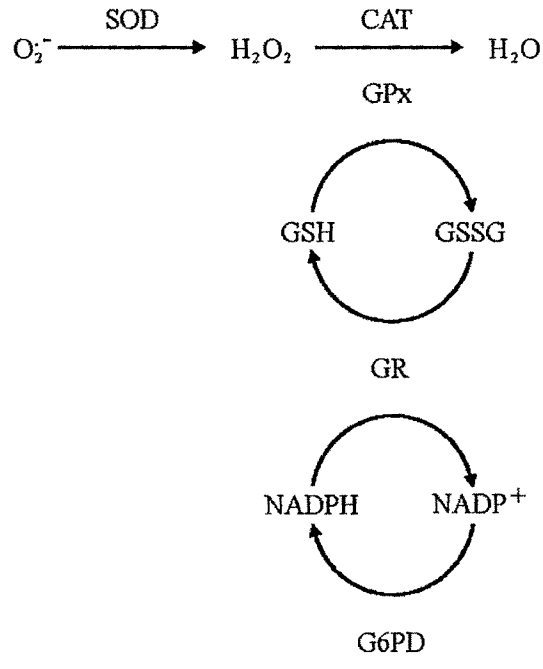
Glutathione is a tripeptide (g-L-glutamyl-L-cysteinylglycine) that is the reductant for glutathione peroxidase. Oxidation of the cysteine sulfhydryl groups joins two glutathione (GSH) molecules with a disulfide bridge to form glutathione disulfide (GSSG). NADPH-dependent glutathione reductase catalyzes recovery of glutathione. Normally, the brain maintains a high ratio of GSH/GSSG for antioxidant defense. Depletion of total glutathione and a decreased GSH/GSSG ratio are markers for oxidative stress in ischemic brain and as long as 7 h may be required to restore concentrations to normal values following an ischemic insult[214,215]. Ischemic outcome is worsened by pharmacological depletion of glutathione[216].

The reperfusion injury following cerebral ischemia in the rat results in loss of GSH[217]. The depletion of GSH is incidentally accompanied by increase in the levels of

thiobarbituric acid reactive substances (TBARS) and reactive oxygen species. Principal consequence of GSH loss during oxidative stress in the brain is the formation of protein glutathione mixed disulfides (PrSSG) and loss of protein thiols[218]. The loss of GSH and formation of PrSSG in the brain results in dysfunction of various membrane functions in cells, such as inhibition of Na+K+-ATPase activity and mitochondrial enzyme activities.

Myocardial hydrophilic antioxidants like GSH were oxidized first in ischemic reperfused myocardial tissue. In myocardium, GSH functions as a substrate of glutathione peroxidase, GSH plays a pivotal role in removing H_2O_2 and preventing generation of ROS. GSH acts as peroxynitrite scavenger, and the reaction of GSH with peroxynitrite forms an S-nitrosothiol compound that regenerates ·NO over a prolonged period[219].

In this reaction reduced glutathione (GSH) is used as a cosubstrate to metabolize H_2O_2, resulting to H_2O and oxidized glutathione (GSSG). Oxidized glutathione (GSSG) can be reduced back to GSH by the enzyme GSH reductase (GR), a reaction requiring NADPH regenerated by glucose 6-phosphate dehydrogenase (G6PDH). The capacity to recycle GSH makes the GSH cycle a pivotal antioxidant defense mechanism for cells and prevents the depletion of cellular thiols.



$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

Glutathione reductase which removes GSSG, recycles GSH and makes it available for reuse to avoid the stress injury.

The individuals with GSH deficiency may well experience excessive oxidative injury as RBCs hemolyse and renders the endogenous defense system vulnerable to damage. Glutathione peroxidase overexpression was found to reduce the necrotic and apoptotic cell death in the cerebral ischemia/reperfusion injury. Various hydrophilic antioxidants (GSH and ascorbic acid) were protective on the myocardial ischemia reperfusion injury.

This glutathione peroxidase is vital for protective activity of CuZn SOD overexpression. Catalase and glutathione peroxidase enzymatic activity is important for the conversion of superoxide-generated hydrogen peroxide to water and oxygen[220].

2.3.1.4 Lipid peroxidation as bio-indicator:

Circulating fatty acid levels increase after an acute myocardial infarction or during cardiac surgery, such that during and after ischemia the heart muscle can be exposed to very high concentrations of fatty acids[97]. The detrimental effects of high plasma fatty acid levels on mechanical and electrophysiologic characteristics of the heart after ischemia reperfusion has been recognized for 20 years[97,98]. High plasma fatty acid concentrations have also been shown to increase the severity of ischemic damage in cardiac ischemia, and have also been linked to a depression of mechanical function during aerobic reperfusion of previously ischemic hearts[99]. During reperfusion of the heart after ischemia, fatty acid oxidation can quickly recover and dominate as the source of ATP production. These high rates of fatty acid oxidation contribute to a marked decrease in cardiac efficiency during reperfusion[100]. However, if glucose oxidation is stimulated during reperfusion, a significant increase in cardiac efficiency results, with a parallel improvement in cardiac function[99,100].

Myocardial utilization of fatty acids has also gained clinical relevance. Elevation of free fatty acids in plasma after myocardial infarction is likely due to a surge of catecholamine activity, increasing the incidence of ventricular arrhythmias[221]. Increased glucose concentration reduces the myocardial uptake of fatty acids[222]. Cardiac fatty acid uptake and transport are interlinked to glucose metabolism during the reperfusion injury[223]. Apparently, proteins are involved in the uptake and transport of long chain fatty acids. Membrane proteins are also important for transfer of fatty acids across endothelial and muscle cells membranes. Fatty acids themselves appear to be responsible for the protein interaction. Exogenous fatty acids can act also in cardiac transcriptional activity[224]. Fatty acids are more vulnerable for oxidation by ROS[225]. These ROS further react with polyunsaturated lipids in membranes generating lipid peroxidation products that can inhibit protein synthesis and alter enzyme activities. Oxidation of polyunsaturated fatty acid moieties of membrane phospholipids can cause membrane disintegration, mitochondrial dysfunction, and Ca^{++} overload. It has been shown that altered cellular redox state and increased lipid peroxidation are associated with the transition of cardiac hypertrophy to heart failure[226]. And the measurement of these

peroxidizable fatty acids and lipids can work as a vital indicator for the prediction of ischemia and the following reperfusion injury.

Brain has the liability of more easily peroxidizable fatty acids, which consumes an inordinately higher fraction of total oxygen consumption. Moreover, brain lacks an enriched defensive antioxidant system[225]. Concentrations of free fatty acid concentrations are markedly increased during cerebral reperfusion injury, the largest increase being that of arachidonic acid[199]. As a result, ischemia-induced Ca^{2+} influx and energy failure promote free fatty acid release and concomitant membrane damage. Free fatty acid metabolism has multiple other adverse effects including inhibition of oxidative phosphorylation[227], formation of pro-inflammatory mediators[228], free radical generation and lipid peroxidation-mediated chain reactions[229], and cytotoxicity from lipid peroxidation products[230], which may stimulate cell death in the vulnerable tissue[231]. Increased nitric oxide concentrations associated with ischemia may have dual effects on lipid peroxidation. Reaction of nitric oxide with superoxide causes formation of peroxynitrite that initiates lipid peroxidation *via* reaction of lipids with its decomposition products hydroxyl radical and nitrogen dioxide. But the role of nitric oxide is controversial for its action on lipids. Thus the measurement of formed malondialdehyde[232] as an indicator of lipid peroxidation can be used as a tool to predict the injury at certain extent.

2.3.2 Creatine kinase (CK)

In search of the markers for ischemia and early predictors of infarct, CK was found to be a reliable marker of ischemic injury. Thus the depletion of CK in myocardial tissue can be availed as an index of ischemic cell injury and infarct formation[233]. CK is an important cellular enzyme mediating energy transduction in muscle cells. In myocardium, CK perfects the regulatory mechanism of energy production and utilization by facilitating the high energy phosphates throughout the cell[234]. CK contains the SH groups which are easily oxidisable at the exposure of ROS[235]. Myocardial necrosis is associated with failure of integrity of the myocardial cell membrane that allows escape of intracellular enzymes like CK from myofibrils after the period of ischemia and reperfusion. CK activity proved to be a pivotal cardiac marker due to its rapid appearance and marked increase in serum after acute ischemia[236]. It appears that CK-MB is derived from the necrotic myocardial cells. Free release of CK indicates the cell death. The estimation of CK release is a quantification of infarction and its

correlation with histological and ECG finding establishes the proportionate increase of CK activity to the extent of myocardial damage[238,239]. The rise in CK-MB isoenzyme is a highly specific diagnostic tool in acute myocardial injury.

Creatine kinase (MM and BB) is also found in brain tissue and may be elevated in the diseased condition. Mlinarik et al, (1998)[239] studied the rate constants of the CK reaction in brain after chronic ischemic condition. However, further research is required to establish CK as a reliable marker of cerebral reperfusion injury.

2.3.3 Myeloperoxidase (MPO), a biomarker of inflammatory reactivity:

MPO, a polymorpho nuclear neutrophil derived heme protein, is a biomarker index for neutrophil infiltration and inflammatory reactivity. Role of MPO activity in myocardial reperfusion injury is well established[241,242]. Neutrophil accumulation in the myocardium was measured by determining the activity of MPO, an enzyme specific for neutrophils[243]. Impaired microvascular function during myocardial ischemia and reperfusion is associated with recruitment of polymorphonuclear neutrophils (PMN) and has been attributed to decreased bioavailability of nitric oxide (NO). Whereas MPO facilitates oxidative NO consumption and impairs vascular function in animal models of acute inflammation. Upon activation, PMN not only generate superoxide and H_2O_2 but they also secrete MPO. MPO in the presence of H_2O_2 is anatomically poised to catalytically consume endothelial-derived NO; thereby impairing NO-dependent signaling in smooth muscle cells and subsequent vascular relaxation thus contributes in the endothelial dysfunction.

The molecular mechanism MPO mediated toxicity to cell is halogenation and oxidation. Halogenation involves the covalent binding of the halide co-factor to the target cell or molecule. Among the possible products formed with protein acceptors and iodide as the cofactor are iodotyrosines, iodohistidines and sulfenyl iodides. The presence of these chemical configurations near the active site could cause loss of biological activity of the cells. The oxidation may be mediated by such peroxidase system intermediates as halogens, hypohalous acid, chloramines, aldehydes and singlet molecular oxygen. These MPO components reacts with extracellular halogen and damages the tissue in the vicinity of inflammatory response as the cell free peroxidase system destroys leukocyte, platelets and other mammalian cells[244].

A study by Baldus et al, 2004 reported that the plasma from patients undergoing myocardial reperfusion contained increased levels of MPO, which catalytically consumed

NO in the presence of H_2O_2. Given the correlation between intravascular MPO levels and forearm vasomotor function in patients with coronary artery disease, MPO appears to be an important modulator of vasomotor function in inflammatory vascular disease and a potential therapeutic target for treatment.

Increased brain MPO activity (in pericore region) after transient focal ischemia virtually reflects the neutrophil infiltration and this neutrophil infiltration into the ischemic brain is implicated in post ischemic brain injury (which was evident from the histological observations)[245]. Thus MPO can be considered as a biomarker to indicate the inflammatory response in the reperfusion injury of heart and brain.

2.3.4 Electrographic changes: ST elevation:

It is widely accepted that ST segment elevation is a clear index of myocardial ischemia. Epicardial ST elevation in transmural ischemic condition results when the injury currents flow between the ischemic myocardial regions to normal myocardium[237,246]. ST segment displacement occurs due to two major mechanisms at cellular level; one of the crucial ways is a localized shorting of duration of action potential and the decreased amplitude of action potential, while the other way is related to a localized decrement in resting membrane potential[237]. The present study shows that, the ST segment elevation was evident in all the ischemic animals at the onset of ischemia and was continued till reperfusion, this could be traced back to the subendocardial ischemic injury.

2.3.5 Measurement of plasma fibrinogen:

Epidemiologlcal observations indicate that high plasma fibrinogen levels are strongly correlated with the frequency of two major thrombotic complications of stroke and myocardial infarction. Wilhelmsen et al.,(1984) [247] reported on the synergistic effect of fibrinogen levels and blood pressure on stroke and suggested that high plasma fibrinogen is a risk factor for stroke and myocardial infarction. More recently, other prospective studies[248,249] have given new impetus to these observations and strengthened their clinical relevance. Thus, measuring plasma fibrinogen to predict stroke and myocardial infarction may be important in gaining insight into the thrombogenic potential of this protein and in inspiring new strategies.

2.4 Pharmacological strategies to protect the reperfused tissue:

Maintaining and recovering the reperfused tissue is the foremost goal of the pharmacological interventions carried out during the reperfusion injury. The objective of achieving the protection of the ischemic tissue must be (i) restoration of flow to the tissue as soon as possible, (ii) using interventions to slow the rate of development of ischemic injury until such a time as reperfusion can be affected and (iii) minimising the severity of reperfusion injury so as to facilitate as rapid a recovery of function as possible.

A number of conceptual approaches can be identified to help facilitate the achievement of these goals. These include: (i) the application of anti-ischemic agents to slow tissue injury, (ii) the use of reperfusion interventions to accelerate recovery from reperfusion injury and (iii) the exploitation of endogenous (adaptive) responses[56].

2.4.1 Myocardial reperfusion injury management:

The impetus to the research in developing the appropriate approach for cardioprotection is the lack of reliability and complete cessation of damage caused to the myocardium after the injury. The first objective of the treatment is to assure immediate survival of the patient are to maintain the general comfort and the well-being of the patient, to limit the ischemic damage, to optimally manage associate complications.

2.4.1.1 Anti- ischemic interventions:

These anti-ischemic interventions are designed to target the deleterious ischemia-induced changes. Three basic approaches have been combined and Practiced by surgeons so to greatly enhance the ability of human heart to withstand extended periods of severe ischemia. This has involved: (i) the use of cardioplegic agents such as the potassium to rapidly arrest the heart during ischemia and thereby conserve energy for tissue protection and subsequent recovery, (ii) the inclusion in cardioplegic solutions of agents such as buffers, substrates and drugs to combat as many deleterious ischemia-induced changes as possible and (iii) the combination of chemical cardioplegia with hypothermia so as to further slow the rate of cellular reactions that contribute to ischemia-induced injury.

2.4.1.1.1 Sodium-Hydrogen Antiport Inhibition

Recent research on inhibition of sodium–hydrogen exchange (Na^+–H^+) has received recognition as results showed it may act as a potential cardioprotectant factor.28 Ischemia and reperfusion result in marked intracellular acidosis; this in turn activates the sarcolemmal Na^+–H^+ antiport, which facilitates proton extrusion (in exchange for

Na).3,28 The intracellular hypernatremia that develops results in activation of the sodium–calcium (Na^+ –Ca^{2+}) exchanger, with resultant increases in intracellulat Ca^{2+} load. Indeed, inhibitors of Na^+–H^+ exchange have been demonstrated to exhibit marked cardioprotection in experimental models of ischemia and reperfusion. More recently, the Guard During Ischemia Against Necrosis [GUARDIAN] trial was investicgating around 10, 000 patients for cardioprotection using Na^+–H^+ inhibitor cariporide [29]. The outcome of the trial encouraged the researchers and the physicians to look at Na^+–H^+ inhibitor as a possible tool to achieve the goal of cardioprotection of the ischemic heart.

2.4.1.1.2 Trimetazidine :

Trimetazidine (TMZ) has been found to have cardioprotective effects on ischemic cardiac tissue. During ischemic episodes TMZ maintain cellular ATP levels, limit intracellular acidosis andaccumulation of inorganic phosphate, Na^+ and Ca^{2+} in cardiac tissue. These changes are independent of oxygen demand and supply alterations. TMZ selectively inhibits the fatty acid β-oxidation enzyme: 3 ketoacyl-CoA-thiolase, and lacks direct hemodynamic effects[80]. It also counters ROS generation[251]. In patients with acute and chronic coronary artery disease, TMZ is safe and has antianginal and antiischemic clinical effects[252].

2.4.1.1.3 Inhibition of TNF-α:

Since the original report of elevated levels of TNF- α in patients with CHF, there has been increasing speculation that TNF- α may contribute to the development of this pathology. It has been shown that pathophysiologically relevant concentrations of TNF- α are sufficient to mimic certain aspects of the CHF phenotype, including LV dysfunction and dilation[254]. TNF- α receptor type II (sTNF-RII) on early (1 wk after surgery) cardiac dysfunction and remodeling and late (10 wk after surgery) CHF[255]. A single intravenous infusion of sTNF-RII, which binds to TNF- α and functionally inactivates this cytokine, led to a significant improvement in LV function and remodeling 7 days after ligation in rats. Finally, this therapeutic approach also showed beneficial effects in rats with CHF 10 wk after MI.

TNF- α plays an important the pathological role of after MI in rats. Profound cardiac function depression and cardiac geometric alterations occurred simultaneously with a peak of TNF- α 7 days after coronary occlusion. Moreover, this critical period contributed to the subsequent development of CHF. Thus, although this anti-TNF- α therapeutic strategy has been shown to be ineffective in the treatment of CHF, it could be beneficial in the prevention of this pathology if acute treatment is initiated early after MI[256].

2.4.1.2 Interventions to minimize reperfusion injury and the recovery of post-ischemic injury:

In order to avoid any further complications due to much needed reperfusion various approaches can be used. The ultimate aim of these approaches is to reduce the cell death and recovery of the cells in the region of area at risk. This approach makes it be possible to use agents such as: (i) anti-oxidants to attenuate stunning – an approach which would seem preferable to overriding stunning through the administration of inotropic agents such as calcium or catecholamines with their attendant risk of increasing heart rate and arrhythmogenesis, (ii) vasoactive drugs to improve the extent and quality of reperfusion by manipulating vascular responses during reperfusion and (iii) ion and pH regulation to promote the rapid reestablishment of ionic homeostasis.

2.4.1.2.1 Vasoactive agents and relaxing factors:

ACE inhibitor, captopril, contains a sulfhydryl moiety in its chemical structure. The sulfhydryl structure has also been suggested as a chemical scavenger of free radicals that may contribute to captopril's actions. Several recent reports found Ang-II inhibition beneficial for CVD and nonhemodynamic actions rather than acute vasorelaxant effects mediate its action per se. It has been recognized that Ang-II itself is a stimulator of cardiac and vascular oxidative pathways via induction of NADH/NADPH oxidase. Therefore, inhibitors of the renin-angiotensin system (ACE inhibition or Ang-II receptor antagonism) may actually serve as indirect antioxidants by blocking this pathway, and some aspects of their long-term efficacy may be related to this effect. ACE inhibition recently has been shown to improve endothelial function in patients with coronary artery disease or its risk factors. While the mechanism of this clinical benefit is unclear, inhibition of AngII-mediated superoxide production may be a protective mechanism associated with preservation of endothelial health and vasoactive properties[257].

The vasodilating β-blocker carvedilol has also been shown to possess antioxidant effects in vitro and in vivo. This agent has emerged as a valuable therapeutic drug for long-term survival enhancement in heart failure and in long-term hypertension control. The antioxidant actions of carvedilol (and its primary metabolite) significantly contribute to cardioprotective effects in hypertension and CHF[258]. Carvedilol administration significantly increased the resistance to LDL oxidation in patients with essential hypertension. Inhibition of ROS in the myocardium may prevent the consequences of oxidative damage[259].

2.4.1.2.2 L-arginine

The bioavailability of NO is known to be inversely related to the presence of other ROS. One approach to augment NO bioavailability is through enhanced provision of NOS substrate arginine. Clinical studies have also shown that L-arginine supplement improved coronary and peripheral blood flow in healthy and heart failure patients[260]. However, the excessive NO generation is harmful. NO acts to prevent the endothelium in the ischemic heart and causes the vasorelaxtion[260,261]. Other agents, which reduced the cardiac, work and produce relaxation like niroglycerine and other nitrates can be used.

2.4.1.2.3 Antioxidants

Oxidative stress is now a well established factor in inducing the damage during the reperfusion injury. Numerous antioxidants are tested and tried on animals and humans to attenuate the damage. Few of the antioxidants are discussed here. The hydrophilic antioxidant vitamin C, has recently shown that it activates NOS in endothelial cells[262,263]. A single dose of oral vitamin C supplement (2 g) reduces arterial stiffness and platelet aggregation in healthy subjects[264], and it improves endothelial function in CHF and angina patients[263]. Similarly, Vitamin E along with Lipoic acid appears to be the most effective lipid soluble antioxidant in biological systems. It inhibits lipid peroxidation and regenerates reduced vitamin C and GSH. The cardioprotective effect of oral vitamin E against oxidative damage has been well recognized in both animal studies and clinical trials[227]. Lipoic acid is reported to exert its cardioprotection by normalising oxygen uptake and glucose utilisation. It has been suggested that a 200 showed that a daily dose of Vitamin E (200 IU/kg/day) for 5-10 days achieves around two fold increase of the vitamin in ischemic rat myocardium.

2.4.1.3 Activating endogenous responses:

The cardioprotection can be achieved by exploiting a variety of endogenous adaptive responses, two of which are currently focus of current research attention [13, 14]. These are: (i) the ability of the heart to express 'stress proteins' and (ii) the phenomenon of ischemic preconditioning. The ability of heart to responds to a variety of stresses (for example transient heat shock) by the expression of a spectrum of stress response proteins. These proteins, which are maximally expressed some 24 h after the initial stress, have been shown to be responsible for a substantial enhancement of the ability of the heart to resist ischemic injury[265]. Likewise, the phenomenon of ischemic preconditioning, is an adaptive response whereby the resistance of the heart to injury

during ischemia and reperfusion can be *temporarily* enhanced by prior (less than 2 h) exposure to one or more *brief* (less than 5 min) episodes of ischemia and reperfusion. Myocardial preconditioning has been shown to be able to protect against tissue necrosis (measured as a it has been show to be capable of improving post-ischemic recovery of contractile function (probably as a secondary consequence to the reduction of infarct size) and, in some species, it has also been shown to exert a profound protective effect against both ischemia- and reperfusion-induced arrhythmias. The brief periods of ischemia that precondition the heart can be substituted by a number of pharmacological and biochemical mimetics[4]. Thus, brief exposure to adenosine, norepinephrine, bradykinin and a variety of other receptor agonists can produce striking protection and these interventions can assist in the resolution of the mechanisms underlying this important adaptive phenomenon.

2.4.2 Neuroprotection in cerebral reperfusion injury:

Recanalization is the primary focus during the first hours after onset of ischemic stroke. However, preservation or neuroprotection of ischemic brain tissue by other means, prevention of stroke complications and maximization of neural function of the surviving brain, and the start of therapies to prevent recurrent stroke are also important treatment goals. The molecular events that mediate ischemic brain damage are logical targets for pharmacological intervention and include glutamate accumulation, aberrant calcium fluxes, free radical formation, and lipid peroxidation.

Recanalisation of the blood vessel can be achieved by anti-thrombolytic, fribrinolytic and anti-platelet drugs which are already discussed in the stroke therapy section of introduction chapter. Here, we will focus on the neuroprotective therapy and the new advances in the treatment of Stroke and reperfusion injury.

2.4.2.1 Antioxidants and the therapy based on antioxidant markers:

A crucial role in triggering and maintaining the post-ischemic insult to brain tissues is represented by the oxidative stress which follows the reperfusion phase of stroke. Reperfusion of the ischemic brain, excessive release of excitatory amino acids, such as glutamate, and infiltration by neutrophils are major sources of reactive oxygen species (ROS) generation. These, in turn, amplify the profound imbalance found in the neurons and astroglial cells of the ischemic core and penumbra. Thus, antioxidants are

certainly among the most promising therapeutic class for the treatment of ischemic stroke, and this may probably be due in part to the fact that their therapeutic window may be longer than that of other strategies. Besides being effective alone, these molecules may also have benefit in combination with other therapies considering the various stroke etiologies and the different deleterious mechanisms involved in stroke lesions. Despite the multitude of experimental studies showing a beneficial effect of antioxidant strategies, very few antioxidants have been or are currently tested in clinical trials

2.4.2.1.1 Inhibition of lipid peroxidation:

Numerous pharmacological inhibitors of lipid peroxidation have been tested. The most notable is tirilazad, a non-glucocorticoid steroid. After the first phase II trial (STIPAS:Study of Tirilazad mesylate in Patients with Acute ischemic Stroke), two phase III studies were started: RANTTAS (Randomized Trial of Tirilazad in Acute Stroke) with patients from North America and the parallel trial in Europe, Israel, and New Zealand (TESS, Tirilazad Efficacy Stroke Study). However the results from most of these trials are not significant in reducing the neuronal damage[266].

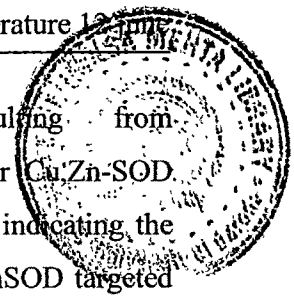
2.4.2.1.2 Inhibition of xanthine oxidase

Allopurinol and oxypurinol were scrutinized for their antioxidant potential. Allopurinol is oxidized by xanthine oxidase to oxypurinol, which binds to the active site of xanthine oxidase causing xanthine oxidase inhibition. Thus, either compound can be administered with the same net mechanistic effect. Allopurinol decreases post-ischemic cerebral uric acid, xanthine and conjugated diene concentrations[267,268], preserves ATP[269], and reduces edema. Deapite thse activities the results for allopurinol in rats are mixed [270], and must be studied further to assess the exact effect of xanthine oxidase inhibitor on brain. The possible reason for this mixed reason could be the lack of its action on superoxide and hydrogen peroxide generation by other routes.

Many herbal agents found to have significant protective activity in stroke. *Curcuma longa*[271] and *Gingko biloba*[272], were studied for its effect on the murine models for stroke and further research is going on these herbs to develop a ideal herbal therapy for stroke.

2.4.2.1.3 SOD and advance SOD mimietics:

The major endogenous SOD, which are studied widely for its effect on ischemic brain are CuZn SOD, MnSOD and ECSOD (extracellular). Both Cu,Zn-SOD and MnSOD are abundant in neural tissue and for this reason have received greatest scrutiny.



Cu,Zn-SOD overexpression reduces ischemic damage resulting from schemia/reperfusion[273]. However, neither Cu,Zn-SOD overexpression nor Cu,Zn-SOD targeted deletion alter the outcome from permanent focal ischemia[274,275], indicating the requirement of reperfusion for this enzyme to play a role. In contrast, MnSOD targeted deletion worsens the outcome from both temporary and permanent middle cerebral artery occlusion[276,277]. Cu,Zn-SOD overexpression has been shown to inhibit post-ischemic injury. EC-SOD overexpressing mice have increased tolerance to both focal and global cerebral ischemia[278], while EC-SOD knock-outs exhibit enhanced damage[279]. Mollace et al, (2003)[280] beneficial effect of M 40401, a SOD mimetic in gerbils.

2.4.2.1.4 Catalase and Glutathione peroxidase acitivity:

Hydrogen peroxide generated is eliminated by catalase and glutathione peroxidase. Both are present in the brain although GPx (glutathione Peroxidase) activity is sevenfold greater than that of catalase. Further, while glutathione peroxidase is present in the cytosol, catalase is localized mainly in peroxisomes. Overexpression GPx reduces necrotic and apoptotic cell death, astrocytic/microglial activation andinflammatory cell infiltration. Ebselen is a synthetic mimetic of glutathione peroxidase[281]. It is not selective in that it also inhibits protein kinase C, 5-lipooxygenase, cyclooxygenase and NADPH oxidase[282]. Thus, inferences from the efficacy of this drug in the context of ischemia/reperfusion regarding the role of glutathione peroxidase are currently being studied in ongoing clinical trials[283,284]. The Japanese Ebselen Study Group performed studies on the efficacy of ebselen in ischemic stroke.

The other two antioxidants on the clinical trial at various phases are Edaravone, a free radical scavenger, which delays infarct and edema evolution and decreased the motrality in acute stroke patients. However the improvement in functional recovery is not significant. NXY -059 is another free radical scavenger, which is studied in different continents and the results are encouraging. Its primary outcome is overall recovery and recovery of motor function[266].

2.4.2.1.5 Nitric oxide synthase inhibitor:

The effect of NOS inhibition was dependent upon which isoform was being inhibited. Pharmacologic eNOS inhibition would be expected to worsen outcome, secondary to cerebral vasconstriction and reduced blood flow. This is supported by studies of eNOS-deficient mice[285] that have worsened ischemic outcomes. In contrast, upregulation of eNOS activity by treatment with 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors (e.g. simvistatin) caused increased intra-ischemic blood flow

and reduced infarct size[286]. Studies confirmed increased peroxynitrite formation occurring in parallel with upregulation of iNOS[287]. Inhibition of iNOS may have protective effect on the ischemic injury in brain.

2.4.2.1.6 Poly (ADP-ribose) polymerase inhibitors

One of the first report indicated that PARP knock-out mice exhibited profoundly diminished cerebral infarct sizes when compared to wild-type counterparts[288]. Poly(ADP-ribose) is synthesized from NAD by PARP and degraded by poly(ADP-ribose) glycohydrolase (PARG). PARP is activated in response to DNA damage as a repair mechanism but also causes NAD and ATP depletion, potentially exacerbating ischemic injury. A principal source of DNA damage is likely to be peroxynitrite formation from superoxide and nitric oxide, mediated by NMDA receptor activation[289,290]. Cu,Zn-SOD overexpressing mice do not exhibit post-ischemic PARP activation (. Effects of pharmacological antioxidants on PARP activation have not been reported. Pharmacological PARP antagonists have provided protection in several ischemia models. Similarly, treatment with systemic NAD improved ischemic outcome[291]. PARP activation remains a conceivable mechanism to explain downstream effects of oxidative stress on ischemic outcome.

2.4.2.1.7 Spin traps application:

Spin trap is a method to 'capture' ROS allowing their detection and quantification. A classic application of this technology in the study of ischemic brain is use of salicylate, which reacts with hydroxyl radical to form a relatively stable adduct, 2,3-DHBA. Taking a different approach, in addition to nitroxide spin probes, nitrone spin traps were developed to capture ROS, allowing detection by electron paramagnetic spectroscopy. Recognizing the potential for nitrones to scavenge ROS, it was considered that these compounds might present therapeutic potential[292]. Indeed, early rodent studies found consistent benefit from the spin trap a-phenyl-N-t-butyl-nitrone (PBN) against both global and focal ischemic insults. More important, the second generation spin trap, NXY-059 (disodium 4-[(tert-butylimino)-methyl]benzene-1,3-disulfonate N-oxide), has been found to improve ischemic outcome in primates when measured at 10 weeks after permanent occlusion of the middle cerebral artery, even when treatment was begun as late as 4 h after onset of ischemia[293]. NXY-059 has been shown to maintain Akt activation and inhibit cytochrome *c* release after ischemia[294].

2.4.2.2 Glutamate antagoniost:

Glutamate induced excitotoxicity is implicated in the reperfusion injury damage. Hence the neuroprotective therapy can avail one more avenue of glutamate antagonists. A new glycine-site NMDA receptor antagonist SM-31900 is a potent neuroprotective agent without adverse effects and indicate that it is a promising therapeutic for stroke. SM-31900 exhibits nanomolar affinity for this receptor site and selectivity relative to other neurotransmitter receptors and ion channels. In rat models of transient and permanent focal cerebral ischemia, postischemic administration of SM-31900 significantly decreased infarct volume, with potency and efficacy superior to gavestinel, another glycine-site NMDA receptor antagonist. Unlike other NMDA receptor antagonists (dizocilpine, selfotel, eliprodil), neuroprotective doses of SM-31900 were not associated with adverse effects, such as psychotomimetic effects, neuronal vacuolization, renal toxicity or cardiovascular effects. (Prous Science, 2006). YM872, a AMPA receptor antagonist, is a neuroprotective compound that ameliorates the deterioration of the hemodynamic penumbra after focal ischemia[295].

2.4.2.3 Inhibition of interleukins:

IL-1 receptor antagonist, Anakinra treatment groups showed neuroprotection, particularly in the cortex, with a significant increase in the frequency of surviving cortical neurons. A nonsignificant reduction in the area of focal infarction in the striatum was also seen in the IL-1ra-treated groups[296].

2.4.2.4 Matrix metalloproteniases inhibitors:

Evidence implicating the involvement of matrix metalloproteinases (MMPs) in the disintegration of the vasculature and BBB disruption following cerebral ischemia. Investigators at University of California at San Diego examined the effects of pharmacological inhibition of MMPs with the relatively nonspecific inhibitor **batimastat** (BB-94; British Biotech) in a rabbit model of tPA-induced hemorrhage after thromboembolic stroke. This study reported that pretreatment with an MMP inhibitor may improve the safety of tPA in the management of acute stroke, preventing intracerebral hemorrhage, although further studies are necessary[297].

2.4.2.5 Heat shock proteins:

HSP70 and HSP72 are expressed as strongly or in one case much more strongly, in CA1 than in CA3 and dentate granule cells. Neurons in the penumbra, many of which recover, show a marked HSP70 production. Thus the correlation between HSP and resistance to damage is present in focal ischemia. Expression of the protein inhibits the activation of the transcription factor NF-κB, which is activated in ischemia and appears to be associated with toxicity. This might well be the protective mechanism; it has not been studied explicitly. The 70-kDa HSP also protects against apoptosis, by blocking the downstream actions of caspases on activation of PLA_2, the cell nucleus, and other processes. This would certainly be a protective mechanism in ischemia[161].

Although many therapeutic agents showed potential promise in animal models, the results of most single-agent clinical trials were sobering. Consequently, various authors advocated studies to evaluate the efficacy of combined therapeutic approaches.
In the present studies, we have studied various agents and evaluated their effects on reperfusion injury alone or in combination with various hormones. The selection of the agents is based on the antioxidant properties exhibited by the drug in various reported studies.

2.5 Role of Hormonal Replacement Therapy (HRT) in reperfusion injury:

The use of sex hormones to prevent or treat neurobiological and cardiovascular (CVD) diseases has become a highly debated topic because of the recent clinical findings using HRT[298,299]. These clinical studies were initiated on the basis of the extensive experimental data indicating the possible effect of hormones in treating injury. The reports of gender difference in the HRT treatment induced more interest in this research area. Among the alleged risks of estrogen and progesterone in HRT, its effects on CVD and stroke have been the most reviewed topics. It is now well established and widely accepted that CVD and neurological diseases are the major cause of death in women as well as in men, although women are more affected at a later age may be after the menopause[300]. Interestingly, there are numerous reports of cardioprotective and neuroprotective effects of estrogen and progesterone combination and estrogen alone treatment in the MI and stroke incidences[301-304]. Estrogen is extensively studied for its age and sex related effects on acute myocardial insult[305]. The effect of progesterone on acute

myocardial I/R injury is not extensively studied. Progesterone is reported to have protective effect on cerebral I/R injury in rats[204,306].

However, numerous studies reported the conflicting observations about the susceptibility of males and females to the cardiovascular and cerebral disorders, however, this susceptibility may be directly or indirectly interlinked to the sex hormones[308-311]. There many reasons for the observed discrepancies in HRT studies, like the route of administration of hormone, dosage of hormones and the age of the target population.

The major aspect all these studies is to balance the reports of action of HRT and to develop a therapeutic plan that will provide the prevention and protection in the patients at risk of CVD and stroke.

The antioxidants are certainly among the most promising therapeutic class for the treatment of ischemic stroke and myocardial reperfusion injury, and this may probably be due in part to the fact that their therapeutic window may be longer than that of other strategies. The combination of these antioxidants with hormones may be effective in treating and managing reperfusion injury. Besides being effective alone, these molecules may also have benefit in combination with other therapies considering the various stroke and myocardial reperfusion injury etiologies and the different deleterious mechanisms involved in reperfusion induced lesions. Of interest is that combination therapies might decrease dosages for each agent, and consequently reduce the occurrence of adverse effects.

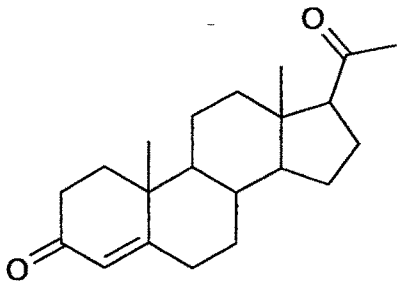
2.6 Progesterone (PG):

Reproductive hormones, such as estrogen, progesterone, and testosterone, are synthesized from a common cholesterol precursor pathway. Specifically, gonadal steroidogenesis starts with cholesterol, which is converted to pregnenolone and progesterone, both precursors of androstenedione and androstenediol, which are in turn converted to testosterone and, finally, to estradiol[312].

Biosynthetic pathways of progesterone

The synthesis of PG from cholesterol involves two enzymatic steps: the conversion of cholesterol to pregnenolone by cytochrome P450scc and the conversion of pregnenolone to PG by 3β-hydroxysteroid dehydrogenase (3β-HSD). Cytochrome P450scc cuts the side-chain (scc,or side-chain cleavage)of six carbons (C_{22}-C_{27}) from the cholesterol molecule, and this reaction takes place in the mitochondria[313].The rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane is a

limiting step in pregnenolone formation and involves two mitochondrial membrane proteins: the peripheral benzodiazepine receptor (PBR; also named mitochondrial benzodiazepine receptor) and the steroidogenic acute regulatory protein (StAR)[314].The mechanism by which PBR and StAR allow the intra-mitochondrial transport of cholesterol is still not completely understood[315,316]. It is important to note that the PBR is distinct from classical benzodiazepine sites, because it is not associated with $GABA_A$ receptors, and it recognizes selective ligands such as the agonist Ro5-4864 and the antagonist PK11195. Pregnenolone moves from the mitochondria to the microsomal compartment, where it is converted to PG by 3β-HSD. The full name of the enzyme,which can use many 3 b hydroxysteroids as substrates, is 3β-hydroxysteroid dehydrogenase/D D isomerase because it catalyzes two reactions on separate active sites located on a single protein: the dehydrogenation of 3β-hydroxysteroids (3β-HSD activity)and the subsequent isomerization (ketosteroid isomerase [KSI]activity)of D 3-ketosteroids (double bond between C5 and C6) to D 3-ketosteroids (double bond between C4 and C5)[317].



Progestrone

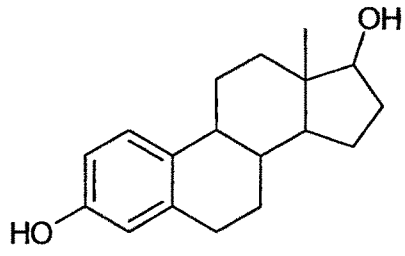
Fig. 1 Structure of Progesterone

PG is further metabolized to 5α-dihydroprogesterone by the steroid 5α-reductase (two distinct iso-zymes) and to 3α- 5α- tetrahydroprogesterone (3α- 5α TH PG, allopregnanolone)by the 3α-hydroxysteroid oxidoreductase (3α-HOR). Both PG and 5α-dihydroprogesterone bind with high affinity to intracellular PG receptors (PRs) and activate gene transcription. In contrast, 3α- 5α TH PG does not bind to PRs, but it is a potent positive modulator of $GABA_A$ receptors. In females, the ovaries are the main source of circulating PG, except during pregnancy, when the hormone is produced by the placental syncytotrophoblasts. The adrenal glands also contribute significant amounts of circulating PG in rodents and humans. Thus, in men, circulating PG is exclusively of adrenal origin[318]. PG produced by the gonads and adrenal glands easily crosses the blood brain barrier and rapidly accumulates within nervous system tissues.

The effects of PG are mediated by its nuclear receptors. These receptors are composed of two receptor proteins, PRa and PRb, each of which binds to PG. PG receptors are practically present in every system of the body. PG receptors are found in many of the same brain areas as estrogen receptors, including the hypothalamus and limbic system. The limbic system, particularly the amygdala, plays a prominent role in regulating emotion and mood. PG decrease brain excitability, whereas estrogens increase it. PG is also synthesized by Schwann cells and enhances myelin repair in the brain[319]. PG exerts various effects on endothelium, vessels and cell memebrane. These effects are attributed to explicit activity of PG on inflammatory mediators, NOS and free radicals[36,319].

2.7 Estrogen:

In addition to its role as a sex hormone, estrogen affects the structural and functional CVS and CNS. Estrogen is synthesized by ovary in female and in small amount by the testis in male and by the adrenal cortex in both the sexes. It also influences the development of, growth, differentiation, maturation and function of various tissues through out the body.



17 -beta estradiol

Fig. 2 Struture of 17- beta estradiol

The starting material for estrogen synthesis is cholesterol. The immediate precursor of estrogen and the androgenic substances are androstenedione or testosterone. After the metabolism of cholesterol to androstenedione via prenenolone, aromatase catalyses the formation of estrone. There are three main endogenous estrogens in humans i.e. estradiol, estrone and estriol. The biological activity of estrogen is mediated the nuclear receptors, ERα and ERβ. ERα and ERβ are the products of two different genes that are located on separate chromosomes. From a functional perspective, ERs have three main domains- the amono terminal or A/B domain, the DNA binding or C domain, and

the ligand-binding or E domain. The F domain is involved distinguishing between estrogen agonist and antagonist[303].

Estrogen also has been reported to possess other events compared to untreated postmenopausal important cardioprotective properties by influencing the lipid profile, Williams et al. (1990)[320] reported estrogen augments endothelial dependent vasorelaxation and possesses calcium channel blocking properties. The ability of estrogen to increase the bioavailability of the vasodilator, anti-aggregat-ing and anti-proliferative substance nitric oxide (NO) and contribution to endothelium and its relaxation factors (NO, PGI_2 and EDHF) causes vasorelaxation of perfused heart and provides the cardioprotection. Estrogen reported to exibit free radical scavenging property[321].

Estrogen treatment to physiologically relevant plasma levels improves histological, physiological, and behavioral outcomes after transient middle cerebral artery occlusion[322]. Estrogen causes neuroprotection by receptor dependent and receptor independent mechanism. It leads to transcription of critical gene that promotes cell survival by action of ERα receptor. The other potential neuroprotective mechanisms are following: endothelial effect that induces vasodialation; antioxidant activity; various receptor mediated actions which activates the survival pathway. The interaction with $bcl\text{-}_2$ proto-oncogenes (involved in cell survival) and neuronal growth factor are important for protecting neurons from the damage. Alteration of GABA neuronal activity, stimulation of neuronal regeneration and secretion of neurotransmitter are the other feature of estrogen which accounts for its neuroprotective activity[323].

2.8 Lipoic acid: A Dual Antioxidant

Lipoic acid (thioctic acid) is 1,2-dithiolane-3-pentanoic acid, is a molecule which contains two thiol groups and 1,2-ditholane ring, first isolated from liver samples in early 1950s. Lipoic acid and di-hydrolipoic acid, its reduced form are the natural constituent of biological membranes, acting as mitochondrial lipoamide co-factor of α-cetoacid de-hydrogenase. Lipoic acid is highly protein bound by amide linkage to a lysine residue; this linkage is called as Lipoyl group, which plays a lead role in many multienzyme complexes.

Alpha-lipoic acid is unique in its ability to act as an antioxidant in fat- and water-soluble tissues in both its oxidized and reduced forms. It is also readily absorbed from an oral dose. Because of these advantages and its low toxicity, alpha-lipoic acid is receiving increased attention as a potentially effective therapeutic agent in clinical conditions

associated with free radical damage. Lipoic acid is reported to be an excellent antioxidant in studies. It is easily absorbed and taken up by the cells. The exogenous α-lipoic acid is rapidly reduced in liver to di-hydrolipoic acid by enzyme lipoate de-hydrogenase using NADH as co-factor. NADH and NADPH systems play a major role in conversion of lipoic acid intracellularly. Di-hydrolipoic acid, the reduced product of lipoic acid produced in intracellular spaces acts as antioxidant in extracellular space. An antioxidant function for alpha-lipoic acid was discovered in 1959 by Rosenberg and Culik, who reported that, Lipoic acid prevented both scurvy symptoms in vitamin C-deficient guinea pigs and vitamin E deficiency in rats fed a diet lacking a-tocopherol[324-326].

Lipoic acid is studied for its antioxidant property in various models of ischemia and reperfusion injury. Lipoic acid found to have antioxidant effect in cerebral ischemia reperfusion injury owing to its ability of improving survival and protecting the rat brain against reperfusion injury following cerebral ischemia[327]. The mitochondria are the main site of antioxidant action of α-lipoic acid. The lipoic acid increases the intracellular glutathione levels; it also has the significant influence on ion Fe^{2+} chelation, nitric oxide and other reactive oxidative species scavenging. S-enantiomer of lipoic acid was more effective than the R-enantiomer when administered 1 hour before ischemia. Lipoic acid administration attenuates liver ischemia reperfusion injury (IRI) via PI3-kinase/AKT pathway which plays a pivotal protective role in IRI.. Lipoic acid pretreated organs reduces postischemic activation of NF-kappa Band activating protein-1 (Muller, 2003).To addition of its protective antioxidant effects, Haramaki et al, (1993)[328], proposed that lipoic acid improves the cardiac recovery after ischemia. In myocardial tissue of diabetic rats, higher doses of alpha-lipoic acid initially normalized glucose uptake and utilization, and consequently normalized oxygen uptake, myocardial ATP levels, and cardiac output, while a low dose of alpha-lipoic acid normalized lactate and pyruvate production. In a type II diabetic model using insulin-resistant obese Zucker rats, alpha-lipoic acid found to increase the uptake of glucose in the absence of insulin[329].

Alpha-lipoic acid should not be given in high doses to patients suspected of having a thiamine deficiency unless the thiamine deficiency is also corrected. High doses of alpha-lipoic acid administered intraperitoneally to rats are reported to cause fatal complications. The prior administration of thiamine prevents these adverse effects. Researchers are in general agreement that alpha-lipoic acid is capable of scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, but not hydrogen peroxide, peroxyl, and superoxide[330]. Di-hydrolipoic acid is both an antioxidant and prooxidant in

studies where hydroxyl radicals were generated. It protects against singie strand DNA breaks induced by singlet oxygen, although it does not do so directly and several steps might be involved in the process[331].Lipoic acid is rightly so regarded as a dual antioxidant owing to its pro-oxidant effect at higher doses and very effective antioxidant action at low doses along with its characteristic ability to act as an antioxidant in fat- and water-soluble tissues in both its oxidized and reduced forms.

2.9 Co-enzyme Q-10:

A lipid soluble Benzoquinone, coenzyme Q10 (CoQ), is an essential component for electron transport in oxidative phosphorylation of mitochondria (mitochondrial respiratory chain). Also called ubiquinone, its principal function is to act as an electron carrier between the NADH and succinate dehydrogenases and the cytochrome system[332]. During mitochondrial electron transport, ubiquinone also occurs as semiquinone and ubiquinol, the fully reduced form of ubiquinone. Semiquinone has a role in the generation of superoxide anions during mitochondrial respiration[333], whereas ubiquinol functions as an intracellular antioxidant, presumably by preventing both the initiation and propagation of lipid peroxidation[334,335]. CoQ appears to be involved in the coordinated regulation between oxidative stress and antioxidant capacity of heart tissue. CoQ an endogenous compound held a special position in the intramembrane metabolism due to redox reaction at the benzochinone ring. Localised between Complex I and Complex III in the intramitochondrial space is able to act together with a-tocopherol regenerating an active form of vitamin E from the tocopheroxyl radical complex.

When the heart is subjected to oxidative stress in various pathogenic conditions[336], the amount of CoQ is decreased, which triggers a signal for increased CoQ synthesis. A previous study showed that CoQ treatment produced higher baseline ATP and phosphocreatine concentrations in the rat heart[337]. CoQ is an antioxidant that can be regenerated and is capable of protecting cell structures from oxidative damage during reperfusion[338]. Sugawara et al., (1990)[339] reported that CoQ synthesis decreases during ischemia, myocardial CoQ depletion normally occurs during ischemia and reperfusion. CoQ has a potential antioxidant effect in brain by reducing lactate concentration, inhibiting lipid peroxidation and improves recovery of brain metabolism in ischemic condition[54,340].

The antioxidant actions of CoQ are not limited to the mitochondria but are applicable to any other cell membrane containing CoQ[335]. These properties make CoQ an

ideal therapeutic agent to reduce myocardial and cverebral ischemia-reperfusion injury[338,341].

2.10 Trimetazidine:

Trimetazidine, 1-(2,3,4 trimethoxybenzyl) piperazine hydrochloride is a proven anti-ischemic drug in myocardial I/R injury[342]. TMZ is found to exert its effects without alteration of hemodynamics of heart[343]. TMZ selectively suppresses mitochondrial 3-Ketoacyl CoA thiolase (3-KAT) thereby reducing the free fatty acid metabolism and balancing the cardiac metabolism. These actions control the oxidative stress, prevents I/R induced damage to mitochondrial respiration and exerts an anti-ischemic effect in hearts subjected to ischemia[344,345]. TMZ is reported to preserve the cellular homeostasis by controlling intracellular acidosis and calcium overload in the cells there by conserving the valuable ATP stores to meet the energy requirements[344]. The administration of TMZ is found to have beneficial effects on antioxidant enzymes in brain by inhibiting initiation of glutathione peroxidation and improving levels of thiol components in the neuronal cells[346].

The pathogenesis of cerebral ischemia and myocardial ischemia leading to array of critical cellular changes has few similarities. These changes in cellular and mitochondrial levels are responsive to TMZ[347,348]. Likewise, TMZ is lipophilic and crosses blood brain barrier[349] and hence, TMZ may work as a neuroprotective agent in cerebral I/R injury. The metabolic effects of TMZ during the ischemic period essentially consisted of attenuation of intracellular acidosis, reperfusion was accompanied by an acceleration of the resynthesis of phosphocreatine and a larger reconstitution of the ATP pool. Hence, one of the possible mechanisms of action of TMZ is protection of ATP-producing processes, probably at the mitochondrial level[350].

2.11 *Lagerstroemia speciosa* L (Banaba):

Banaba (*Lagerstroemia speciosa L.* Fam. Lythraceae) is a traditional medicinal herb in Southeast Asia and widely used to treat diabetes mellitus and other aliments. Role of Banaba in obesity and diabetes has been extensively studied[352]. Banaba leaves contain various therapeutically important active constituents like corosolic acid[352] and lagerstromein[353]. Although critical evaluation for clinical use has not been reported, a recent study showed that the extract from the leaves decreases the blood glucose level of genetically diabetic (KK-AY) mice. As the basis of the hypoglycemic action, the Banaba

extract is shown to increase the glucose uptake of 3T3-L1 adipocytes[296]. In efforts to identify the active ingredients, a triterpene, corosolic acid, was isolated from the methanol extract of the Banaba leaves as an activator of glucose uptake of Ehrlich ascites tumor cells. The hypoglycemic effect of corosolic acid in experimental animals has also been reported. Another biologically active compound contained in the banaba leaves is lagerstroemin. The compound increases the glucose uptake of rat adipocytes, physiological target cells of insulin. However, it is not known whether the insulin-like action of lagerstroemin is elicited solely on the glucose transport system or broadly on the insulin-regulated cellular events. The cardioprotective and neuroprotective potential of Banaba is not yet explored.

Fig.3 Photograph of *Lagerstroemia speciosa* L. (Banaba) plant.