Introduction and Aim of the Study

1.1 Ischemic Reperfusion Injury

Restriction or reduction of blood supply to any vital organ of the body leads to deprivation of oxygen and can result in to severe ischemic condition. Restoration of the blood supply to this ischemic region is the pre-requisite for the salvage of the cells at risk. Paradoxically, this delayed recirculation of the ischemic region leads to deleterious effects which include the activating the cascade of events leading to energy deficits, inflammation and ultimately resulting in irreversible tissue injury. The extensive cell injury due to this late replenishment of the ischemic tissue or organ is termed as reperfusion injury.[1,2] The cell death that occurs during reperfusion can be reversible or irreversible depending upon the duration of ischemic insult. This cell death can be characterized microscopically by extensive swelling which may include disruption of tissue lattice, contraction bands, mitochondrial swelling and calcium phosphate designs in mitochondria [3].

Ischemic reperfusion injury can occur to any of the major organs in the body due to various circumstances. But the two most vital organs, which are highly susceptible to reperfusion injury, are brain and heart. The transient periods of ischemia are encountered in the clinical situations of angina, coronary vasospasm, and balloon angioplasty, and are not associated with concomitant myocyte cell death[4,5]. However, with increasing duration and severity of ischemia, greater cardio-myocyte damage can develop and this along with a predisposition to a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury[6]. Reperfusion injury results in myocyte damage through myocardial stunning, microvascular and endothelial injury, and irreversible cell damage or necrosis[6,7]. Stroke or cerebral reperfusion injury occurs due to development of obstructions in small vessel or large artery that ultimately lead to neuronal cell death can arise by six distinct processes: atherosclerosis, embolus, thrombus, reduced systemic pressure, hemorrhage, and vasospasm[8].

In most of the developed and developing countries, cardiovascular diseases and stroke accounts for nearly 2/3 of the total mortality and morbidity cases. Every year about 32 million individuals suffer acute coronary and vascular events and atleast half of these occur in people with established coronary heart disease (CHD) and cerebrovascular disease (CVD). The survivors of myocardial and cerebral infarction are at more to recurrent CHD and CVD than that of normal people. Struke can lead to recurrence in survivors at about 7% per annum, while recurrent myocardial infarction increases the annual death rate by five to six times higher than those who do not have CHD[9].

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States. Approximately 500 000 people suffers from stroke afflicts each year and places a substantial burden on the national healthcare system, which costs an estimated 23.2 billion dollars to national coffers. The alarming mortality rate from stroke is 150 000 deaths per year. These statistics have propelled the search for neuroprotective therapies to reduce cell death and infarct volume after stroke. The rehabilitation of the stroke patient is crucial for the complete recovery. Stroke is also the major cause of adult disability, because 76% of people survive their stroke. This long-term disability means that $30 billion of the $53.5 billion annual dollar cost of stroke is incurred in supporting long-term survivors. Thus, stroke is a lethal disease, but it disables more than it kills. These facts and figures gave an impetus to recent efforts to develop strategies for neural repair and rehabilitation after stroke[10].

In this section some of the aspects of reperfusion injury encountered in crucial-clinical emergencies like stroke and myocardial ischemia are discussed,

1.2 Pathological conditions, which may lead to myocardial ischemia

Myocardial ischemia can occur as result of various vascular and metabolic disorders, which affect the heart directly, or indirectly causing the hypoxic conditions. The damage to heart is directly proportionate to the severity and duration of ischemic insult. The degree and onset of reperfusion greatly influence the outcome the final necrotic injury to the cell.

1.2.1 Metabolic Syndrome

The metabolic syndrome is a cluster of abnormalities, which include abdominal obesity, high blood glucose/impaired glucose tolerance, dyslipidemia, and high blood pressure. These abnormalities together increase the risk of diabetes mellitus and cardiovascular diseases[11]. The patients with higher risk of the metabolic syndrome are highly susceptible to develop myocardial infarction (MI). The individual risk components of metabolic syndrome can greatly contribute to the cardiovascular emergencies.

1.2.1.1 Diabetes

Diabetes, especially Type-2 diabetes is associated with considerable cardiovascular disease (CVD) risk and mortality. Numerous clinical studies proved that the diabetic heart is more sensitive to ischemia/reperfusion injury. However, the individuals with diabetes without prior myocardial infarction have an equivalent risk of coronary heart disease (CHD) to nondiabetic individuals with prior MI. Glucose intolerance which is the characteristic feature of the Type-2 diabetes plays a pivotal role in coronary artery diseases[11]. Diabetes proves to be a risk factor for poor revascularization of coronary arteries in ischemic conditions[12]. Progression of diabetes alters neutrophil adhesion, elicits endothelial and myocyte dysfunction, and exacerbates oxidative stress and dynamics of myocardial energy mechanism, each of which alone or as a cluster may be responsible for the exaggerated reperfusion injury following ischemic insult[13,14]. Contractile dysfunction which predominantly observed in diabetic patients could be a consequence of diabetic cardiomyopathy[15]. The reperfusion injury which results due to various factors during diabetic condition can be explained by array of mechanisms and in nut shell these mechanisms are explained below, a) altered ca^{2+} ion homeostasis and reduced intracellular pH, b) hyperglycemia induced elevated inflammatory mediators like TNF-α and IL-6 in circulation influences the cellular damage, c) reactive oxygen species generation make he myocardium vulnerable to free radical induced injury, d) alterations in the production of nitric oxide, and e) unregulated K_{ATP} channels relinquishes its cardioprotective property. All these factors exert varied responses to ischemia/reperfusion injury encountered in diabetic heart[16,17].

1.2.1.2 Dyslipidemia

Any abnormal change in plasma lipid profile or lipoproteins precipitates into dyslipidemia. Elevated cholesterol and LDL levels are associated with increased risk of coronary artery diseases and development of myocardial ischemic injury[18]. Dwindling NO release from vascular endothelium is a hallmark of elevated serum cholesterol levels in dyslipidemic patients. This decreased NO release may be responsible for endothelium dependent vascular reactivity which potentially leads to microvascular dysfunction [19]. The microvascular dysfunction can cause atherosclerosis by playing a crucial role in its pathophysiology. This micro-vascular dysfunction greatly influences the progression and consequences of ischemia/reperfusion injury. In the heart, a depleted amount of NO exacerbates the ischemia/reperfusion injury due to an increased inflammatory reactivity possibly by accumulation of leukocytes in the myocardium. Oxidative stress plays a major role in hypercholesterolemic heart. Reactive oxygen species, mainly superoxide is excessively generated by arterial endothelial cells exacerbates myocardial ischemia/reperfusion injury[20].

1.2.1.3 Hypertension

Hypertension encompasses the complex mesh of causative factors and chronic elevated blood pressure is the primary symptom of this highly intrusive syndrome. Role of hypertension in ischemia/reperfusion injury is not yet well known but it immensely contributes to ischemic coronary events probably by activating renin-angiotensin system[21]. Clinical studies on patients with ischemic heart disease demonstrated the protective effects of ACE inhibitors and Angiotensin II receptor blocker by improving LV function and preventing geometric remodeling. These events ultimately reduce the cardiovascular complications after ischemia/reperfusion injury[22,23]. Hypertension and oxidative stress are closely related events. Angiotensin II contributes to reperfusion injury by instigating inflammatory cascade and generating the reactive oxygen species. Hypertension enhances the inflammatory responses induced by reperfusion injury and thereby leaves the microvasculature more prone to extensive damage from ischemia/reperfusion injury[24].

1.2.2 Aneurysm

Aneurysms are abnormal dilations of arteries caused by weakening of the arterial wall. Common causes include hypertension, atherosclerosis, infection, trauma, and hereditary or acquired connective tissue disorders. Aneurysms are usually asymptomatic but can cause pain and lead to ischemia, thromboembolism, spontaneous dissection, and rupture, which may be fatal. Aneurysm in coronary arteries leads to myocardial ischemia due to reduced perfusion of the cardiac myocytes. Syphilitic aneurysms of the aortic root classically lead to aortic regurgitation and inflammatory stenosis of the coronary artery ostia, which may manifest as chest pain due to myocardial ischemia[25].

1.2.3 Aortic Dissection

Aortic dissection is the surging of blood through a tear in the aortic intima with separation of the intima and media and creation of a false lumen. The intimal tear may be a primary event or secondary to hemorrhage within the media. Hypertension is an important contributor. This dissection in coronary artery may lead to myocardial ischemia. But serious consequences are common: compromise of the blood supply of tributary arteries (including coronary arteries), aortic valvular dilation and regurgitation, heart failure, and fatal rupture of the aorta[26].

1.2.4 Coronary Spasm

Coronary artery spasm is a transient, focal increase in vascular tone, markedly narrowing the lumen and reducing blood flow; symptomatic ischemia may result. Marked narrowing can trigger thrombus formation, causing infarction. Spasm can occur in arteries with or without atheroma. In arteries without atheroma, basal coronary artery tone is probably increased, and response to vasoconstricting stimuli is probably exaggerated. The exact mechanism is unclear but may involve abnormalities of nitric oxide production or an imbalance between endothelium-derived contracting and relaxing factors. In arteries with atheroma, the atheroma may cause local hypercontractility; proposed mechanisms include loss of sensitivity to intrinsic vasodilators like acetylcholine and increased production of vasoconstrictors (eg, angiotensin II, endothelin, leukotrienes, serotonin, thromboxane) in the area of the atheroma. Recurrent spasm may damage the intima, leading to atheroma formation. Use of vasoconstricting drugs (eg, cocaine, nicotine) can trigger coronary spasm. Spasm-induced MI may occur in normal or atherosclerotic coronary arteries[27].

1.2.5 Acute coronary syndrome (ACS)

Acute coronary syndromes result from acute obstruction of a coronary artery. Consequences depend on degree of obstruction and range from unstable angina to non-ST-segment elevation MI (NSTEMI), ST-segment elevation MI (STEMI), and sudden cardiac death. The symptoms of ACS are chest discomfort with or without dyspnea, nausea, and diaphoresis. Diagnosis is by ECG and the presence or absence of serologic markers.

ACS usually occurs when an acute thrombus forms in an atherosclerotic coronary artery, which activates platelets and the coagulation cascade and produces an acute thrombus. Even atheromas causing minimal obstruction can rupture and result in thrombosis, which may abruptly interferes with the myocardial blood flow. Initial consequences vary with size, location, and duration of obstruction and range from transient ischemia to infarction. Anterior infarcts are usually caused due to left coronary artery obstruction, especially in the anterior descending artery. Transmural infarcts involve the whole thickness of myocardium from epicardium to endocardium. Subendocardial infarcts usually involve the inner ⅓ of myocardium. These infarcts may follow prolonged hypotension. Ischemic and necrotic cells are incapable of normal electrical activity, resulting in various ECG changes (predominantly ST-T abnormalities), arrhythmias, and conduction disturbances. The disturbances in conduction can reflect damage to the sinus node, the atrioventricular (AV) node during the ischemic condition[27].

1.3 Pathophysiological condition responsible for progression of cerebral ischemia

Cerebral infarction may result from one or more pathophysiological mechanism, which includes atherosclerosis, cardioembolism, reduced systemic pressure, hematological disorder and metabolic disorder. The diseases of large arteries and small vessels along with channel disorders can also play vital role in initiation of cerebral damage.

1.3.1 Atherosclerosis:

Atherosclerosis is a pathological process in which calcified lipid or fatty deposits from the flowing blood accumulate circumferentially along the innermost intimal layer of the vessel wall leading to hardening of the arteries. Plaques develop especially at bifurcations of major arteries including carotid artery. The biochemical processes like oxidation of low-density lipoproteins, formation of fatty streaks, and the proliferation of smooth muscle cells causes formation of plaques, harding of wall and narrowing of lumen thus obstructing the blood flow. This obstructing of blood flow leads to turbulence in blood and increases kinetic energy, which dislodges the plaque in blood stream. This plaque lodges in a vessel having smaller size, thus preventing further passage of blood flow. Atherosclerotic thrombosis accounts for 33% of all stroke cases[8].

1.3.2 Cardioembolic stroke:

Cardiovascular dysfunction leads to ischemic stroke. The common disorders are familial arterial myxomas, hereditary conduction defects and hypertrophic obstructive cardiomyopathy[28].

1.3.4 Reduced systemic pressure:

Various mechanisms of blood flow obstruction leading to stroke occur along localized regions of the cerebral arteries. Cardiovascular diseases such as atrial fibrillation and myocardial infarction weaken the cardiac wall and introduce abnormalities in the physiological function of the heartbeat, which ultimately result in reduced systemic pressure and conditions of ischemia[8].

1.3.4 Hematological disorders:

Stroke is a major complication of sickle cell disease. Several mechanisms are suggested for the increased risk of ischemic cerebral stroke in patients with sickle cell disease; these include sickling of red blood cells during a crisis and thrombotic infarction of both large and small arteries[28].

1.3.5 Small vessel disease:

Small vessel disease refers to the structural changes in the small penetrating end arterioles supping blood to deep white matter and basal ganglia. Lacunar stroke syndrome is the most common clinical phenotype of this malformation. These lacunar infarcts may lead to cognitive impairment and disorder of gait[8].

1.3.6 Vasospasm:

When bleeding occurs in the subarachnoid space, the arteries in the subarachnoid space can become spastic with a muscular contraction, known as cerebral vasospasm. The contraction from vasospasm can produce a focal constriction of sufficient severity to cause total occlusion[29]. The length of time that the vessel is contracted during vasospasm varies from hours to days. However, regardless of the duration of vessel constriction during vasospasm, reduction of blood flow induces cerebral ischemia.

1.4 Treatment strategies for acute ischemic condition:

Increased understanding of the biological implications of ischemic injury led to development of various treatment strategies to limit the extent of injury in the subject. The primary objective of the treatment is to assure immediate survival of the patient and to restrict the damage in critical ischemic condition.

1.4.1 Management of acute myocardial ischemic condition:

After initial hospitalization and clinical evaluation, the patient is subjected to specific therapy. This specific therapy of acute myocardial infarction revolves around the preservation of myocardium from injury. The early interventions are designed to limit infarct size by three different approaches viz. a) reduced cardiac work, b) improve myocardial perfusion and c) improve substrate delivery. However, these interventions must be initiated within 4-8 h of commencement of ischemic condition for maximum reperfusion and benefit.

1.4.1.1 Reduction in cardiac work:

Using various Beta-adrenergic blocking agents and nitrates, particularly when chest pain or hypertension is present can reduce the cardiac load during the ischemia. Nitrates especially, nitroglycerine acts by vasodialation via NO release and provide much needed reprieve to the myocardium from excessive cardiac load and O_2 demand. Other vasodilators like calcium channel blockers are also prescribed for the acute myocardial ischemic condition[2].

1.4.1.2 Improvement in myocardial perfusion:

Reperfusion is the most effective therapy for the acute ischemia ever known and studied. The myocardial perfusion can be improved by using drug therapy or with surgical interventions. Once the assessment severity of coronary artery lesions is over prompt revascularization can be performed by percutaneous intervention (PCI) or coronary artery bypass graft (CABG) surgery. Thrombolytic therapy can be used by intracoronary or intravenous routes of administration in the ischemic heart. The earliest intervention in the current therapy appears to be the intravenous administration of streptokinase or urokinase. Tissue plasminogen activator (tPA) is also a potent candidate for this therapy. The anti-platelet drugs like aspirin and clopidogrel (GP IIb/IIIa inhibitor) are also available for use in the ischemic conditions. More recently the abciximab (a monoclonal antibody of GP IIb/IIIa) is a better option in the cases where aspirine or ticlopidine is contraindicated. Low molecular weight heparin or unfractionated heparin is given routinely unless contraindicated (eg, by active bleeding or use of streptokinase or anistreplase)[11,12,30].

1.4.1.3 Improvement in substrate delivery:

Glucose-insulin-potassium solutions have been used because of their ability to counter the elevation of free fatty acid release during acute infarction and to provide substrate for anaerobic glycolysis. Hyaluronidase is used, as it is another source of substrate, which acts by hydrolyzing the hyaluronic acid. Corticosteroids and synthetic anti-inflammatory agents have been shown to reduce the infarct size but these agents may increase the incidences of myocardial rupture. Hypoxia encountered during ischemia can be dealt with administration of high oxygen mixtures to the patient in the early hours of the therapy.

1.4.2 Management of acute ischemic stroke:

The presence of a focal neurological deficit and a history of abrupt onset of symptoms in the absence of trauma suggest the onset of a stroke. Restoration of nutritional blood flow to threatened tissue before they progress to infarction, reperfusion therapies salvage penumbral tissue, reduce infarct size, and enable the improvement in neurological outcome. General treatment includes the administration of supplemental oxygen tissue and isotonic intravenous fluid and anti-thrombotic/fibrinolytic therapy.

1.4.2.1 Intravenous thrombolytics:

Tenekteplase (TNK), a genetically modified form of tPA provides more selectivity for fibrin and greater resistance to inhibition of plasminogen activator type 1. The long lifetime of TNK allows it to administer in single bolus dose and its specificity to fibrin ensures complete clot lysis. Desmetoplase is 1 of 4 distinct proteases found in the saliva of the blood-feeding vampire bat *Desmodus rotundus*, referred to as *D rotundus* salivary plasminogen activators (DSPAs). Desmetoplase is the α-1 variant among the DSPAs, induces faster recanalisation than tPA and has shown promise in phase 2 ischemic stroke trial. Reteplase is a recombinant-peptide that consists of kringle 2 and protease domains of human tPA and works as a potent recanalising agent. Moreover, GP IIb/IIIa antagonists have the ability to dissolve platelet-rich clots and to improve flow in coronary and cerebral microcirculation[30].

Intra-arterial thrombolytic therapy can be performed with a microcatheter that is placed into, beyond, and proximal to an arterial occlusion. Urokinase is conventially used while intaarterial tPA and prourokinase have been extensively studied in recent investigations.

1.4.2.2 Combined pharmacological approach:

The combination therapy is put forth to expand the fibrinolysis time beyond 3 h. The combination therapies studied are anti-thrombotic (Reteplase) and fibrinolytic (Abciximab) provided faster, consistent and sustained reperfusion. Treatment with neuroprotective strategy (hypothermia) with thrombolytic (tPA) agent was promising. Neuroprotectives stabilizes threatened tissue and extends the time window for the subsequent administration of reperfusion agent. The other neuroprtective strategies used in ischemia are glutamate receptor antagonist, calcium channel inhibitors and antioxidants. Administering agents that are vasoprotective (matrix metaloproteinase inhibitor batimastat) along with reperfusion interventions (tPA) may reduce hemorrhagic transformation rates, improve the benefit/ risk ratio, and increase the permissible time window for reperfusion therapy[30].

1.4.2.3 Sonothrombolysis

Experimental and clinical studies have consistently demonstrated the capability of ultrasound (US) to enhance enzymatic thrombolysis. US application increases the transport of tPA into the thrombus, promotes the opening and cleaving of the fibrin polymers, and improves the binding affinity of tPA to fibrin (Molina and Saver, 2005).

Most of the strategies discussed above are aimed at providing the prompt and complete reperfusion of the severely ischemic tissue. Reperfusion of an ischemic area may result, however, in paradoxical cellular dysfunction, a phenomenon termed "reperfusion injury"[2]. In past, most research has concentrated on mechanisms causing cellular injury during ischemia and designing the procedures to reduce the ischemic damage. Very few strategies are to date developed clinically to effectively counter the reperfusion injury. Our study is aimed at investigating few approaches to treat and manage the reperfusion injury.

In the last two decades, the research on reperfusion injury is revolving around the concept of oxidative stress management and the restoration of the metabolic balance of the cells prone to injury[31,32]. Various herbs[33,34], hormones[35,36] and chemical agents[37] were experimentally evaluated for its effect on reperfusion injury. Few of these agents were found to be effective but none of these could be taken to the next step of developing an ideal therapy for the reperfusion injury. However, some of the clinical trials were conducted for the effect of estrogen and progesterone effect on myocardial reperfusion injury[38,39].

1.5 Effect of hormones on reperfusion injury:

Estrogen and progesterone were studied for experimental reperfusion injuries in animals. There are conflicting reports about the effect of estrogen on reperfusion injury. Few researchers found, estrogen to be protective in myocardial as well as cerebral injuries[35,40] while others found that it has deleterious effect on reperfusion injury in animals[41,42]. Clinical trial results were also showing the same trend, as Stefanick (1999) and Gradi et al, (2002) [43,44] reported that effects of hormones were either harmful or ineffective on the patients of reperfusion injury. While, Rosano and Panina (1999)[45] and Cerquetani et al, (2001)[46] showed protective effect of sex hormones in individuals with reperfusion injury. Progesterone was rarely evaluated experimentally in myocardial reperfusion injury, whereas it showed neuroprotective activity in cerebral reperfusion injury. Progesterone was reported to exacerbate the cerebral injury in ovariectomised female rats[47]. In the light of all these contradictory reports of sex hormone's effect on reperfusion injury, the activity of these hormones must be reassessed.

1.6 Role of antioxidants in reperfusion injury:

Oxidative stress generated by free radicals and related reactive oxygen species (ROS) mediate much of the damage that occur during ischemia/reperfusion injury[48,5]. Oxidative stress associated with ischemia reperfusion injury can damage every major cellular component, including membrane lipids, proteins, carbohydrates, and DNA[31]. Antioxidants are exogenous and endogenous compounds acting in several ways including scavenging of ROS, inhibiting ROS formation and chelation of ions needed for catalysis of ROS generation. Various endogenous antioxidant supplementations found to be beneficial in restoring the antioxidant enzymes (SOD, GSH px) during reperfusion injury[49,50]. However, the exogenous supplementation of Lipoic acid[37,51,52], Co-enzyme Q-10[53,54] was found to be protective in the reperfusion injury induced animals.

Therefore, the present study was designed to evaluate the effect of theses sex hormones and their combinations with various antioxidants on the reperfusion injury of brain and heart. This study would further help us to clarify the controversies such as liberation of free radicals and other mechanism which are involved in ischemia/reperfusion and also the usefulness of hormones and antioxidants in reperfusion injury.