CHAPTER 1

INTRODUCTION

Chapter Highlights

- ***An overview of the research problem***
- ***Thesis Objectives and Contribution***
- ***Thesis Organization***

1.1. An overview of the research problem

Cerebrovascular disease remains as the third largest reason behind the global mortality and is also associated with high morbidity and disability [1,2]. About 70% of the cases of cerebrovascular disease involves cerebral ischemia reperfusion injury or CIRI [1]. Besides causing deaths, CIRI also contributes to disability of the survivors leading to economic and psychological stress [1]. Reperfusion or restoration of blood flow in the brain is a major treatment strategy for the cerebral ischemia [1,2], a disorder which is reported to constitute a major percentage of strokes occurring in United States (Fig. 1.1) [3]. Stroke is caused by a deficit of oxygen in brain due to loss of blood supply in a particular region or the whole brain [3]. This may be may be due to blockage in cerebral arteries, known as ischemic damage or due to a burst in arterial blood vessels causing disruption in blood supply, defined as hemorrhagic damage (HS) [3]. Cerebral ischemic damage is widely accepted to be 10 times more prevalent than the haemorrhagic damage of brain [4-16] and can be further classified into thrombosis and embolism (Fig. 1.3). While thrombosis is caused by *in-situ* formation of blood clot leading to arterial narrowing and obstruction of the blood flow in brain tissues [17], embolism is often caused due to sudden blockage of an artery by

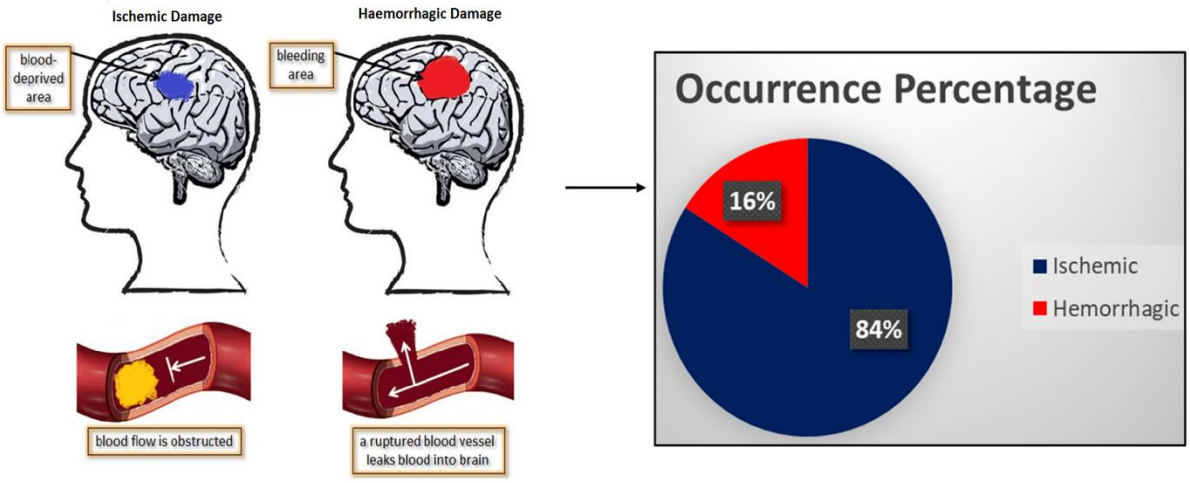

Fig. 1.1. Differentiation between ischemic and hemorrhagic brain damage and respective occurrence percentages globally (IS: 84%; HS: 16%).

a clot that travels, after being formed elsewhere in the body, and lodges itself in the artery to disrupt blood to cerebral regions [17]. This leads to cerebral dysfunction and widespread formation of infarction in the brain region which is not receiving oxygen supply via the blood vessels.

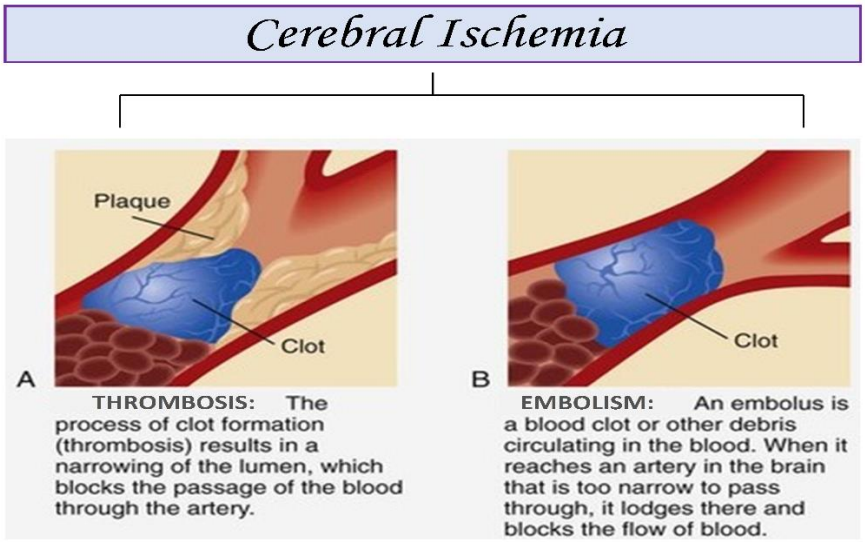

Fig. 1.2. Broad categorization of cerebral ischemic damage

Due to an obstruction in the cerebral blood flow during cerebral ischemia, there happens to be a lack of supply of oxygen and nutrients in brain [18] which further causes death of neurons, disrupts the neuronal synapses and leads to loss of oligodendroglia and astroglia [19]. Hence, restoration of blood flow remains the major goal for combating the ischemic injury in brain [1,2]. But it has been reported that a restoration of the blood flow or reperfusion, following an ischemic insult, often exacerbates the ischemic damage and increases the chance of aggravating the ischemic pathophysiology [1,2]. This phenomenon is commonly known as CIRI [1], which causes an influx of increased concentration of oxygen, thus leading to formation of reactive oxygen species (ROS) which further generates the strongly oxidative peroxynitrite ion by combining with Nitric oxide (NO), which is released due to Nitric oxide synthase (NOS) activation [20]. The excessive oxidative and nitrosative stresses cause neuronal damage [21], induction of DNA damage and onset of various cell death pathways [22]. Though apoptotic cell death has been associated in cerebral ischemia, but caspase-independent cell death pathways mediated by poly- ADP- ribose-polymerase 1 (PARP1) and receptor interacting protein kinase-1 (RIPK1) have been reported as an intrinsic component of cerebral ischemic cell death [22-24].

Even though scientific research has shed considerable light on the cellular, molecular and biochemical processes involved in cerebral ischemia, the heterogeneous nature of this neurological disorder has obstructed designing of safe and efficient neurotherapeutics [25]. Till date, the only Food and Drug Administration (FDA) approved drug used as therapeutics in cerebral ischemic damage, remains the intravenous administration of recombinant tissue-plasminogen activator (rtPA) [26]. Small therapeutic window (4.5 hrs)

is the main limitation of rtPA along with its threat of converting the ischemic damage into cerebral hemorrhage [27]. Due to lack of proper therapeutic strategies in management of cerebral ischemic injury, the present scenario necessitates neuroprotective approaches with ability to interrupt the cell death cascades and thereby reducing the cerebral infarction [28]. Neuroprotection involves inhibition of molecular mediators of ischemic cascade and various scientific studies have established PARP-1 and RIPK1 as potential therapeutic targets [24, 29, 30]. Compounds inhibiting these molecular mediators might have promising approaches in designing of future neurotherapeutics.

From past few decades, phytochemicals from different herbal plants, vegetables and fruits have been studied widely for their neuroprotective abilities [31, 32]. In the Asian countries, nearly 120 medicinal herbs have been traditionally used to treat the disorders associated with central nervous system (CNS) [33]. In the recent years, scientific studies have identified many such herbs which are used traditionally as neuroprotectants against brain injury, neurodegenerative disorders and other neurological disorders [34]. Particularly in India, various Ayurvedic plants like *Nicotiana tabaccum* (Tobacco) [35], *Curcuma longa* (Curcumin) [36, 37], *Ginkgo biloba* (Kew tree) [38], *Bacopa monnierae* (*Brahmi*) [39] and *Withania somnifera* (*Aswagandha*) [40] have played prominent role in scientific studies involving neuroprotection. One of the major constituents of *W. somnifera* is the steroidal lactone Withanolide A (WA), which reportedly exhibited neuroprotective effect in Alzheimer's disease [41], regeneration of damaged synapse and neuritis [42], ageing and daily stress [43]. Though WA is reportedly a neuroprotectant, no study till date reports its ability in combating the pathophysiology induced by cerebral ischemia.

In the present study *W. somnifera* phytochemicals are analyzed using virtual screening tools to identify the possible potent inhibitors of PARP-1, which is reportedly associated with neuronal cell death of cerebral ischemia. WA was *in-silico* identified as a potent PARP-1 inhibitor and further studied *in-vivo* for its neuroprotective ability in cerebral ischemia. It was confirmed that the phytochemical can reach the brain via-intranasal delivery and is able to confer neuroprotection in global cerebral ischemia.

Besides phytochemicals, endogenous hormones like estrogens and prolactin have prominent roles in neuroprotection [44-47]. Lesser volume of infarct size was observed in female rats after 2 hours of middle cerebral occlusion (MCAO), as compared to male rats of similar age groups and physiological conditions [48], suggested the neuroprotective ability of female sex hormones, especially estrogens, in cerebral ischemia [49]. Pretreatment with estradiol has been reported to confer neuroprotection in hippocampal neurons after ischemic insult and to improve cognition in global cerebral ischemia [50]. Estetrol (E4), a major estradiol metabolite, has been recently reported to exhibit neuroprotection in neonatal hypoxic–ischemic encephalopathy (HIE) [51]. Role of E4 in alleviating temperature rise during menopausal hot flushes, a vasomotor symptom, signifies its effect on CNS [51] and establishes its candidature for being evaluated as a neuroprotectant in cerebral ischemia. The estrogen molecules were screened virtually for their ability to inhibit RIPK1 and the the *in-silico* studies revealed E4 as a potent inhibitor for RIPK1. Based on the results of the *in-silico* studies, which suggested that E4 might interfere with necroptosis, a cell death pathaway involved in cerebral ischemia, the molecule was further studied *in-vivo* in global cerebral ischemia model in mice. *In-vivo*

studies revealed that E4 is a strong neuroprotectant capable of preserving the BBB, reducing the cerebral infraction and restoring the cerebral neurotransmitter levels.

Prolactin (PRL), though mostly associated with pregnancy and lactation, is a versatile hormone and acts as a neuropeptide within the brain [52]. PRL is able to crosse BBB and is also produced in the hypothalamic region of the brain [53]. PRL causes neurogenesis [53], provides neuroprotection during stress [54] and controls maternal behavior [55]. Neuroprotection by PRL in hippocampal region against kainic acid induced excitotoxic injury [56] and glutamate excitotoxicity in primary hippocampal cells [57] has been already reported, but no studies till date has established the effect of PRL administration *in-vivo* in combating cerebral ischemia induced pathophysiological conditions. The present study focuses on the effect of intra-nasal administration of PRL on brain damage, physiological and biochemical changes induced due to global cerebral ischemia.

1.2. Thesis Objectives

The principle objective of this thesis is to identify the suitable inhibitors against the molecular mediators of the ischemic cascade and the studying *in-vivo* efficacy of the identified compounds in global cerebral ischemia model. In this light, following objectives are listed below:

1. *In-silico* identification of potent PARP-1 inhibitors from the *W. somnifera* phytochemicals by using molecular docking tools.
2. Evaluation of brain penetration ability of Withanolide A (identified as a PARP-1 inhibitor) and its efficacy in *in-vivo* neuroprotection against global cerebral ischemia.
3. *In-silico* analysis of Estrogens for their ability to inhibit RIPK1, the key molecular mediator of the necroptosis pathway of cerebral ischemia.

4. *In-vivo* evaluation of neuroprotective efficacy of Estetrol, identified as a possible potent inhibitor of RIPK1, in global cerebral ischemia in mice model.
5. Evaluation of neuroprotective potential of Prolactin against global cerebral ischemia in rodent model.

1.3. Thesis Contribution

The thesis contributes in the area of neuroprotection against global cerebral ischemia by endogenous hormones and Withanolide A, a phyto-steroid. The main contributions are listed below:

1. Virtual screening of *W. somnifera* phytochemicals in search of a potent PARP-1 inhibitor. The *in-silico* study further identifies Withanolide A as a potential neuroprotective molecule for combating cerebral ischemic condition.
2. Brain penetration ability of Withanolide A after intra-nasal administration was confirmed and neuroprotective ability of the phytochemical was established in global cerebral ischemia model of mice.
3. Estrogen molecules are *in-silico* evaluated for their ability to inhibit RIPK1. Estetrol is identified as a potent inhibitor of necroptotic pathway based on of its ability to inhibit RIPK1.
4. Neuroprotective ability of Estetrol is ascertained in the global cerebral ischemia model of mice.
5. *In-vivo* confirmation of Prolactin induced neuroprotection in rodent model of global cerebral ischemia.

1.4. Thesis Organization

The remaining thesis has been organized in the following manner to provide detailed information regarding the ideas that has been mentioned before handed:

Chapter 2 presents literature review and theoretical background of cerebral ischemia. The chapter also discusses the ischemic pathophysiological pathways, types of cerebral ischemia, therapeutics and the animal models in cerebral ischemia. Besides, brief overviews of Withanolide A, Estetrol and Prolactin are also discussed. The chapter also covers details of molecular docking techniques and intra-nasal drug administration.

Chapter 3 discusses *in-silico* screening of *W. somnifera* phytochemicals and identification of Withanolide A as an inhibitor of PARP-1. The chapter interprets the binding efficiency and interaction patterns of Withanolide A with PARP-1 catalytic domain and compares the same with reported PARP-1 inhibitors.

Chapter 4 details the brain penetration ability of Withanolide A after intra-nasal administration. *In-vivo* efficacy of Withanolide A in ameliorating global cerebral ischemia induced brain damage is also discussed in this chapter.

Chapter 5 represents virtual screening of Estrogen hormones using molecular docking techniques, thereby identifying Estetrol as a potent RIPK1 inhibitor and an inhibitor of necroptotic pathway. The chapter includes illustration of binding mechanisms and interaction patterns of Estetrol with catalytic domain of RIPK1 and also compares the same with the reported RIPK1 inhibitors Necrostatin-1 and Necrostatin-4.

Chapter 6 contains an in-detail discussion of neuroprotective ability of Estetrol in animal model of global cerebral ischemia. The chapter discusses effect of Estetrol on cerebral infarction, brain tissue and BBB damage and elevated neurotransmitter levels resulting due to global cerebral ischemia.

Chapter 7 discusses the effect of the neuropeptide Prolactin in ameliorating physiological and biochemical changes in rat model of global cerebral ischemia. The

chapter also discusses Prolactin's role in restoring brain tissue damage and BBB breakdown.

Chapter 8 summarizes the findings and contributions of the thesis and highlights major achievements. The chapter also discusses scope of work in future directions based on the studies performed till now.

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