1. Introduction

Two out of every three individuals diagnosed with cancer today, survive for at least 5 years (American Cancer society, US). Epidemiological studies suggest that, one in every three persons will develop cancer at some point of time in their life (Cancer Research 2007, UK). However, drug discovery in cancer therapeutics has made immense contributions to find novel chemotherapeutic drugs that have led to remarkable improvement in neoplastic disease outcomes.

In US, approximately 14.5 million people were alive as on Jan 1st, 2014 who had history of some form of cancer. The expected number of new cases in 2015 is 1.65 million. Five year relative survival rate for all forms of cancer diagnosed during 2004-2010 was found to be 68%. This has increased from 49% during 1975-1977 (AmericanCancerSociety, 2015). The estimated new cases of breast cancer (BC) in 2015 are 2,34,190 out of which forty thousand deaths were expected to occur in females. Death rates due to breast cancer in particular have gradually decreased since 1989 with greater declines in younger women than elder population. It was also noted that death rates due to childhood cancers have declined by 67% over the past four decades. The 5, 10 and 15 year relative survival rates for all the stages of breast cancer combined is now at 89, 83 and 78%, respectively (AmericanCancerSociety, 2015) which is attributed to the advances in cancer treatment strategies and also early diagnosis with improved mammography screening techniques.

As per the latest WHO’s GLOBOCAN-2012 report, BC is the second most common cancer in globally, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). BC is the most common cancer in women in India and accounts for 27% of all cancers in women (Ferlay J and Forman D, 2012). Hence India need to pay attention to BC screening programs for early detection and treatment to improve survival rates. It is also alarming that, incidence of BC in the early 30’s of age in younger woman.

Indian council for Medical Research (ICMR) has started National Cancer Registry Programme (NCRP) which collects the information regarding the number of new cancer cases and mortality cases. Estimated number of people living in India with cancer was around 2.5 million and every year, new cancer patients registered was over 7 lakh (Nandakumar, 2009). Total Indian cancer cases are likely to go up from 979,786 cases in the year 2010 to 1,148,757 cases in the year 2020. Cancer of breast alone was expected to cross the figure of 100,000 by the year 2020 (Takiar et al., 2010).
Although, the survival rates have improved to a great extent, the quality of life (QOL) in cancer survivors has been prominently compromised due to the unavoidable side effects of cancer therapeutics (Ahles and Saykin, 2007; Myers, 2009). Also, adverse effects of chemotherapeutic drugs limits efficacy of chemotherapy in reducing cancer burden. One such side effect was behavioural/cognitive dysfunction which is progressively gaining importance and becoming a major concern in survivors in spite of their longer survival rates (Nelson et al., 2007).

Evidence showed that a subset of cancer survivor population is facing cognitive changes following chemotherapy particularly in breast cancer survivors (Silberfarb et al., 1980). This chemotherapy-induced cognitive impairment (CICI) is popularly known as “Chemobrain/Chemofog/Mentalfog” (Bender et al., 2006; Schagen et al., 2001). Incidence of chemofog complications in survivors ranges from 35-75% in various clinical reports, depending on the type of cancer or chemotherapy regimen employed to treat cancer patients and also variation in diverse neuropsychological testing procedures applied to assess cognitive function in cancer survivors (Myers, 2009; Wefel et al., 2004).

Chemotherapy-induced cognitive impairment, i.e. chemobrain comprises deficits of diverse components of memory ranging from episodic, visual, verbal, language, spatial and working memory to impaired executive function with reduced information processing speed (Bender et al., 2006; Joly et al., 2011). The incidence of chemobrain was highly reported particularly in breast cancer survivors due to their prolonged survival and hence the feasibility or the likelihood of longer follow-up studies in this population (Ahles and Saykin, 2002; Brezden et al., 2000; Olin, 2001; Wefel et al., 2004).

Although, cognitive changes persists for one or two years following chemotherapy and slowly deteriorates as reported by some studies (Schagen et al., 2002), many survivors continue to report cognitive problems for 5-10 years and in some cases, the memory deficits can even last throughout life (Ahles and Saykin, 2002; Burstein, 2007; Hess and Insel, 2007; Myers, 2009).

Further, it was noted that cognitive deterioration is dose dependent, i.e. higher the dose employed to treat cancer, higher the incidence rate of cognitive complications (van Dam et al., 1998). Due to impaired cognitive function, survivors find utmost difficulty to perform day to day activities and lose their independency which has a negative impact on QOL (McDonald and Saykin, 2011).

Various hypotheses were proposed for chemotherapy-induced cognitive dysfunction viz., myelosuppression, reduced glucose uptake, diminished blood flow and metabolism (Silverman et al., 2007), increased oxidative stress, dysregulated immune response (Myers, 2010), chronic neuro-inflammation (Aluise et al., 2011), inhibition of adult hippocampal neurogenesis (AHN)
(Monje and Dietrich, 2012), white matter degeneration (Deprez et al., 2011; Stemmer et al., 1994), blood brain barrier disruption which can lead to neurotoxicity and cognitive impairment in cancer survivors (Ahles and Saykin, 2007; Seigers and Fardell, 2011; Simó et al., 2013). Some chemotherapeutic drugs, viz. cyclophosphamide, 5-flourouracil can easily cross blood brain barrier while others (paclitaxel, DOX etc.) cannot. Further, it was found that to induce chemofog condition, crossing the BBB is not a prerequisite. The agents which may not effectively cross BBB can produce chemofog through an indirect mechanisms of neurotoxicity, viz. release of inflammatory mediators (cytokines, IL-6, IL-8, TNF-α etc.) which can easily cross BBB and induce neuro-inflammation, microglial activation, mitochondrial dysfunction with an eventual neuronal cell death.

From the previous reports on clinical studies in cancer survivors, it is difficult to find the causal relationship between chemotherapy and cognitive impairment. This is due to the occurrence of high variation from patient to patient in view of disease status, selected chemotherapy, emotional distress to cancer diagnosis, other treatments and differences in cognitive tests applied (Vardy et al., 2007). Further, it is also difficult to quantify the extent of memory impairments reported. However, it was found that the complaints from the patients are more serious and frequent than what has been observed in clinics (Boykoff et al., 2009; Munir et al., 2010).

The confounding factors that were obvious in clinical studies can be avoided by making use of suitable animal models which can reflect the clinical chemobrain complications. To date, no interventions are found to be effective to alleviate the symptoms of chemofog associated cognitive deficits although some agents were tested clinically. A recent review reported that several clinical or preclinical studies worked on interventions viz., modafinil, methylphenidate, ginkgobiloba, resveratrol, N-acetyl cysteine, NSAIDS etc. However, they were not completely effective to alleviate the cognitive complications (Davis et al., 2013; Fardell et al., 2011).

Further, we noticed a major research gap that no suitable and relevant animal models were available which could reflect the cognitive complications associated with chemobrain condition as seen in cancer survivor population. Hence there exists a great need for developing suitable chemofog animal models to explore cognitive impairment objectively following the cytostatic treatment and also to identify possible mechanisms underlying the chemobrain phenomena. Exploring novel therapeutic interventions to prevent cognitive complications is also a need of the hour as there is a major lacuna in this regard.

Doxorubicin (DOX), a broad spectrum anthracycline cytotoxic antibiotic, is used to treat almost all forms (solid and liquid) of cancers and is in use since more than half a century. It is...
the preferred drug of choice for both benign and metastatic forms of cancers, especially for breast cancer of all stages. DOX was given either alone or in combination with other antineoplastic agents for most of the solid cancers and various types of leukemia which can produce toxic effects on brain (Singal et al., 2000). Cognitive impairment was particularly evident in breast cancer survivors who underwent chemotherapy in particular including the DOX based therapies (Schagen et al., 2001). Despite its wide spread use for many cancers, not much attention has been paid for the neurotoxic potential associated with chemobrain complications.

In this current work, we initially tried to develop most suitable and relevant animal models of chemobrain for episodic and spatial memory components with DOX. Some of the earlier reports revealed that cancer patients may have cognitive deterioration before chemotherapy due to emotional distress and agitated response to the cancer diagnosis which may become a confounding factor in assessing the chemobrain related cognitive deficits. To exclude this confounding factor, we designed another study to assess influence of mammary cancer per se on cognitive processing in female rats.

Animal behavioural data support the fact that flavonoids can improve various components of cognitive processes through neuronal differentiation, long-term potentiation and also by enhancing the synaptic plasticity (Spencer, 2007; Spencer, 2009; Wang et al., 2006; Williams et al., 2004). Two such flavonoids are naringin (NAR) and rutin (RUT) containing naringenin and quercetin as pharmacologically active aglycone moieties, respectively. Both flavonoids have wide spread pleiotropic beneficial pharmacological activities viz., antioxidant, antiinflammatory, antiapoptotic, anticancer, antidepressant, myeloprotective, cardioprotective, nephroprotective and neuroprotective effects in-vivo (Kim et al., 2009; Raza et al., 2013).

Since chemotherapeutic drugs used to treat cancer can produce organ related toxicities and various other side effects including neurotoxicity which can be possibly prevented by pleiotropic pharmacological actions of naturally occurring bioflavonoids, we hypothesise that NAR and RUT can be used as possible adjuvant therapeutic interventions to alleviate the cognitive complications associated with chemotherapy. For more reliability and to correlate the animal data with breast cancer survivor population, we tried to establish the chemobrain animal models in female rats rather than in males as the previous work related to chemobrain on male rats is ambiguous and unclear. Furthermore, as the cancer survivors report cognitive changes once they are diagnosed with breast cancer followed by chemotherapy treatment, it is ideal to assess the cognitive function in mammary cancer animals following chemotherapy which is feasible only by using female rats.
Initially, we assessed the validity of using female rats for assessing episodic memory process by testing the cognitive component in both male and female Wistar rats. We also assessed gender difference for the extent and formation of episodic memory in either sex and also assessed spatial memory associated with chemobrain in female rats.

To exclude the influence of cancer on cognitive changes, we initially explored the chemotherapeutic drug, DOX in healthy animals followed by assessment of its chemobrain inducing potential in cancer animals so as to find the most relevant animal model for chemobrain. We selected N-nitroso, N-methyl urea (NMU)-induced mammary cancer which is the most relevant form of human breast adenocarcinoma for correlation of chemobrain complications observed in breast cancer survivors. We successfully developed NMU-induced carcinogenesis in female rats with reproducibility and a short period of tumor latency.

Following the successful chemobrain model development as mentioned above either in healthy or mammary cancer bearing rats, we evaluated the bioflavonoids, i.e. NAR and RUT to alleviate cognitive complications and other side effects associated with DOX. Further, we tested underlying mechanisms involved in conferring protection by these flavonoids against chemobrain by evaluating neuroprotective potential of NAR and RUT in neuronal cell lines. Human neuroblastoma (IMR32) and rat pheochromocytoma (PC12) cell lines were used against DOX-induced neurotoxicity to assess effect of flavonoids by various in-vitro assays viz., cell viability by MTT assay, neuritogenic assay for measuring the neurite outgrowth, detection of apoptosis by AO/EB staining, ROS estimation and cell cycle analysis by flow cytometry etc.

The present doctoral thesis work is designed systematically in an attempt to improve the QOL in cancer survivors through the development of suitable and relevant animal models of chemobrain, so that the potential interventions can be screened preclinically with utmost reliability. Moreover, oncologists can decide, which combination of chemotherapeutic drugs need to be given preference to treat cancers with less incidence of chemobrain complications. The study also assessed possible interventions, NAR and RUT for chemobrain associated with DOX chemotherapy as there are no approved medications.
2. Literature Review

One of the earliest work on chemotherapy related cognitive complications was by Silberfarb who reported that “cognitive impairment to be a common occurrence in the absence of affective disorders or other psychopathology. Chemotherapy was the major variable associated with cognitive impairment in these patients” (Silberfarb et al., 1980).

This very preliminary report explains the state of knowledge existing today, which has been already explored 35 years ago. This first report in the 1980’s also revealed that patients undergoing chemotherapy for various cancers like leukemia, Hodgkin’s disease, digestive and respiratory malignancies etc. performed significantly low on various neuropsychological cognitive tests. The study also identified the fact that permeation of chemotherapeutic agents into the brain was not a prerequisite for inducing chemofog condition that has resulted from a non CNS-directed chemotherapy.

Since this report, there is an increasing interest in this topic as the patients consistently report cognitive problems following chemotherapy that has led to remarkable increase in number of publications in the field of chemobrain/ chemofog till date (Raffa, 2010).

Cytotoxic agents due to their nonspecific action can target normal healthy and rapidly proliferating cells (blood cells, gastric mucosal lining), with common side effects being myelosuppression, loss of hair and specific organ toxicity etc. (Schagen et al., 2002). Although most of the cytotoxic agents do not cross BBB effectively (for e.g. DOX), they still can produce chemobrain associated cognitive dysfunction through their peripheral cytotoxic effects in animals (Joshi et al., 2005). Increased levels of oxidative free radicals like, ROS, RNS and peroxynitrite present in the periphery as a result of chemotherapy can easily cross BBB and are deleterious to normal neuronal cell population as a result of protein and lipid oxidation products such as protein carbonyls, 3-nitrotyrosin and malondialdehyde, 4-hydroxy nonenal etc. (DeAtley et al., 1998).

Further, it was found that, although DOX cross BBB in low levels, the proliferative neuronal death was highest with DOX exposure in animals (Christie et al., 2012) which possibly explains the role of peripheral mechanisms of toxicity that reflects an indirect mechanism of brain damage. No interventions are found to be effective to alleviate the chemofog symptoms due to lack of relevant animal models that can reflect the clinical chemofog condition. In our present study, we have focused on developing the most relevant animal model to reproduce chemobrain like condition for the associated complications of cognitive battery for diverse components of memory viz., episodic, spatial memory etc.
In preclinical studies, most of the researchers have employed healthy male rats treated with diverse chemotherapeutic agents, however the results are highly ambiguous and mixed with no clarity (Seigers and Fardell, 2011). Generally in neurocognitive studies, male rats are preferred over female rats possibly because of lack of influence by estrus cycle. Further, it was reported that male rats learn quicker in complex maze tasks (Jonasson, 2005). However, gender specific cognitive deficits were observed in long-term breast cancer survivors and proved clinically who have undergone chemotherapy in particular (Olin, 2001). Hence, it is more appropriate to develop a behavioural animal model using female rats (regardless of estrus cycle interference) for the cognitive deficits induced by chemotherapeutic agents. Further, the chemobrain studies in mammary cancer rats will be possible only in female rats. For this, we need to assess the gender difference for the extent of memory processes in both male and female rats so as to quantify the difference and establish cognitive models of chemobrain in female rats with more relevance to the clinical chemobrain phenomena seen in breast cancer survivors.

It was found that, chemotherapy induced cognitive dysfunction involves episodic memory impairment in survivors (Rollin-Sillaire et al., 2013), which can be assessed preclinically using object recognition task. Hence episodic memory was studied in much more detail as the day to day activities are most disturbed in chemobrain condition affecting the QOL in survivors. Also this model allows researcher to incorporate the changes according to the timely need/requirement for the model development.

Animal studies on DOX-induced cognitive complications are scarce and very few studies were found which have assessed only emotional memory component (Liedke et al., 2009). Further, in rodent studies, DOX was found to increase the susceptibility of brain mitochondria to calcium-induced permeability transition pore opening and oxidative stress predisposing neuronal cells to degeneration (Cardoso et al., 2008). Hence we have selected DOX as the chemotherapeutic agent to induce chemobrain like condition with possible episodic memory deficits in a validated novel object recognition task in female Wistar rats. This is to correlate the outcome and also the use of female rats as a relevant animal model for chemofog that is associated with human breast cancer survivor population.

2.1. What is chemobrain???

It was found that many of the cancer survivors undergoing chemotherapy as adjuvant therapy reporting cognitive problems to their oncologists which directs for cessation of chemotherapy, hindering use of effective chemotherapy for treating cancer (Ahles and Saykin, 2002; Olin, 2001). The chemotherapy induced behavioural dysfunction is popularly known as Chemobrain/Chemofog/Mentalfog. Chemobrain comprises difficulty in concentration,
impaired executive function, visual, verbal, episodic and working memory deficits with a reduced processing speed (Brezden et al., 2000). The behavioural abnormalities were especially noticed in breast cancer (BC) survivors who underwent chemotherapy in particular due to the remarkable increase in survival rates and the feasibility of longer follow-up (Wefel et al., 2004).

2.2. Chemobrain in cancer survivors
A subset of cancer survivor population (35-75%) was facing the chemobrain complications (Ahles et al., 2002). Often these complications persists over a period of 2 to 3 years and in some cases, it can even last throughout the life.

2.3. Chemobrain in experimental animals
Preclinically many researchers tried to explore the influence of various chemotherapeutic agents either alone or in combinations on diverse memory components in rodents. Most studies reported deficits of one or two memory processes (spatial or emotional forms) on acute treatment with chemotherapeutics in healthy animals (Seigers and Fardell, 2011). All the studies have utilized male rats rather than females due to their quick learning abilities. In contrast, some reports showed no alterations in cognitive function or even some reports revealed positive effects of chemotherapy on cognitive function. The animal experimental data on chemobrain was mixed and ambiguous. Using cancer bearing animals in particular, mammary cancer models can have better correlation with the clinical findings of chemobrain symptoms in BC survivors.

2.4. Chemotherapeutic agents proved to induce chemobrain
Since chemofog condition was noticed in cancer patients who underwent systemic chemotherapy, many studies have explored cognitive side effects of diverse chemotherapeutic agents used clinically. Some reports have revealed that, cognitive dysfunction may result from using specific cytotoxic agents or it may be due to cancer itself (Peterson and Popkin, 1980). A report by Wieneke and Dienst found that, 75% of cancer patients who received standard cytotoxic therapy (DOX, 5FU and cyclophosphamide) have suffered from cognitive deteriorations after 6 months with affected attention, concentration, visuospatial and verbal memories (Wieneke and Dienst, 1995).

Researchers also noticed that, chemobrain effects were dose dependent, i.e. high dose chemotherapy will result in more deleterious CNS side effects than standard dose therapy even after 2 years of post-chemotherapy (van Dam et al., 1998).

A study of breast cancer survivors in 2000 found that, 50% of the patients who have received standard chemotherapy, suffered cognitive complications supporting the earlier reports.
Ahles and Saykin revealed that, the chemobrain is not an imaginary phenomenon and is real, impairments are noticed in cancer survivors upto 10 years following chemotherapy (Ahles and Saykin, 2002).

2.5. Agents other than chemotherapeutics that may produce chemobrain like condition
Researchers reported the influence of other therapeutic regimens used for cancer such as radiotherapy (Shibayama et al., 2014), hormonal therapy and surgery etc. which may contribute to chemobrain complications (Le Rhun et al., 2015), however the most reported cases were noticed following chemotherapy, especially in BC survivors with affected QOL. Researchers reported that emotional distress to cancer diagnosis will have negative influence on behaviour especially, cognitive function well before the initiation of chemotherapy (Berman et al., 2014).

2.6. Adult hippocampal neurogenesis (AHN)
Recently, it was proved that neurogenesis is a continuous physiological process throughout the adulthood and is essential for the formation of new memories. Dentate gyrus (DG) region of hippocampus is the primary site of adult hippocampal neurogenesis. Reports proved that, nearly 700 new neurons were added daily into each hippocampus to form new memory processes (Spalding et al., 2013). DOX was reported to induce inhibition of hippocampal neurogenesis in animal models of chemobrain (Christie et al., 2012). Cytotoxic agents are lack of specificity for cancer cells, so they can be lethal to the newly forming neuronal stem cells. This can result in memory impairment which is recognized as “chemobrain/chemofog”.

2.7. Proposed mechanisms underlying “Chemobrain” (Ahles and Saykin, 2007)
Neurotoxicity is thought to be the key pathological mechanism for the resulting chemobrain complications. Although the exact mechanism underlying chemobrain is yet to be known, there are some proposed mechanisms, viz., myelosuppression, dysregulated cytokines, direct neurotoxicity, increased levels of inflammatory mediators (TNF-alpha), reduced blood flow and diminished metabolic activity in brain, degeneration of white matter, inhibition of adult hippocampal neurogenesis etc.
Fig. 1.1. Possible diverse mechanisms underlying neurobiology of “Chemobrain”

2.8. Bio-flavonoids
Bio-flavonoids are naturally occurring plant derived polyphenolic compounds with wide range of beneficial actions, viz., antioxidant, antidepressant, anticancer, neuroprotective effects etc. which may benefit the cancer survivors to get rid of chemotherapy-induced side effects mainly organ related toxicities as well as the behavioural dysfunction.

2.8.1. Naringin and rutin flavonoids as possible interventions for chemofog
Among the plant derived bio-flavonoids, naringin (NAR) constitutes an important flavanone type of flavonoid glycoside with naringenin (flavanone) as its active pharmacological aglycone moiety. Rutin (RUT) comprises flavon glycoside with quercetin (flavonol) as aglycone part which is pharmacologically more potent. Both the flavonoids have proven neuroprotective potential in various neuronal cell lines in-vitro as well as in-vivo and they are also known for their potential anticancer activity.
NAR, a citrus flavanone glycoside found in grape seeds. NAR possess anti-oxidant, anti-inflammatory, anti-apoptotic and cardio-protective effects in-vivo (Kim et al., 2009; Raza et al., 2013). It is well known that, inflammatory responses and their mediators play a key role in the pathogenesis of neurodegenerative disorders (McGeer et al., 2006). NAR was reported to attenuate behavioural changes and cognitive impairment in kainic acid-induced epilepsy (Golechha et al., 2011) and 3-nitropropionic acid-induced Huntington’s disease animal models (Kumar and Kumar, 2010). NAR also prevented cognitive deficits in colchicine (Kumar et al., 2010a) and D-galactose (Kumar et al., 2010b) -induced learning and memory deficits in rats. NAR was reported to enhance Calcium/Calmodulin dependent protein kinase II (CaMKII) activity, thereby long-term potentiation in rats (Wang et al., 2013). Moreover, NAR showed neuroprotective effects in mouse model of Parkinson’s disease (Kim et al., 2016). Naringin was also found to improve DOX-induced behavioral deficits by modulation of serotonin level and mitochondrial complexes protection pathway in rat hippocampus (Kwatra et al., 2016).

Another flavonoid used in the present dissertation work, i.e. RUT is a flavonol glycoside has diverse beneficial effects viz., hepatoprotective, antioxidant, anticancer, antiplatelet, and antithrombotic, vasoprotective, cardioprotective and also potential neuroprotective activities (Janbaz et al., 2002; La Casa et al., 2000; Pu et al., 2007; Schwedhelm et al., 2003; Sheu et al., 2004). RUT inhibited microglial activation and pro-inflammatory cytokines (Koda et al., 2009); effective against trimethyltin-induced spatial memory deficits through amelioration of neuronal damage in hippocampal CA3, crucial for acquisition learning (Koda et al., 2008). RUT was proved to enhance BDNF gene expression and MAPK pathway in brain, thereby improved the cognitive function against Aβ induced neurotoxicity in rats (Moghbelinejad et al., 2014). An analogue of rutin, troxerutin was reported to enhance synaptic plasticity in the dentate gyrus region of hippocampus of Aβ treated rats (Babri et al., 2014). RUT with exercise found to reverse HFD induced cognitive deficits in obese mice (Cheng et al., 2016). Quercetin, aglycone of RUT was found to enhance LTP as reported by many observers (Yao et al., 2010).
Anti-inflammatory effects of NAR and RUT have been reported in many experimental studies and their role in inhibition of nitric oxide (NO) production was also studied (Fang et al., 2008; Rotelli et al., 2003; Wang et al., 2008). They also showed potential neuroprotective effects against ischemic reperfusion-induced cerebral injury by ameliorating oxidative damage, mitochondrial dysfunction and neurological impairments (Gaur et al., 2009; Khan et al., 2009). Moreover, researchers found that NAR and RUT alleviated AD type neurodegeneration and associated cognitive impairment induced by intracerebroventricularly (ICV) injected streptozotocin for spatial and working memories in rats (Javed et al., 2012; Khan et al., 2012). Hence we selected the flavonoids, NAR and RUT to explore their protective potential against DOX-induced chemobrain for episodic and spatial memory components in the developed animal models.

Fig. 1.2. Possible beneficial mechanisms of flavonoids to alleviate “Chemobrain”
Bibliography


Chapter 1  Introduction and Literature Review


Chapter 1

Introduction and Literature Review


Yao, Y., Han, D.D., Zhang, T., and Yang, Z. (2010). Quercetin improves cognitive deficits in rats with chronic cerebral ischemia and inhibits voltage-dependent sodium channels in hippocampal CA1 pyramidal neurons. Phytotherapy research : PTR 24, 136-140.