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# Nanoencapsulated flavonoid imparts neuroprotection against age-related ischemia-reperfusion induced neuronal damage

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## ABSTRACT

Cerebral stroke, the leading cause of death and permanent disability among the elderly, occurs when blood flow to the brain is insufficient to meet metabolic demand. Cell death occurs days after initial ischemic insults known as the delayed neuronal death. Loss of integrity of the blood-brain barrier (BBB) resulting from ischemia/reperfusion leads to vasogenic brain edema, a primary cause of stroke-associated mortality. Because most neuroprotective drugs do not cross the BBB, it is not unforeseen that most disorders of the CNS could benefit from improved drug therapy. Moreover, decreased endogenous antioxidant defenses with age may further accelerate ischemic injuries. Nanoparticles (NPs), that have been devised nowadays for drug delivery purposes can readily cross the BBB without compromising its integrity, have enormous applications in the treatment of ischemic stroke. Our group devised a long circulating polymeric nanocapsule made of polylactide-co-glycolide (PLGA) which can encapsulate flavonoids efficiently and can cross the BBB and provide sustained drug release avoiding degradation process. Furthermore, PLGA nanoparticles encapsulating flavonoids might play a neuroprotective role against oxidative damage by preventing neuronal loss in different vulnerable brain regions, especially from the hippocampal subfields (CA1 and CA3) thereby increasing the chances of survival in ischemic stroke.

**Keywords:** *quercetin, nanoparticles, reactive oxygen species, ischemia-reperfusion, mitochondria, hippocampus.*

## 1. INTRODUCTION

Cerebral stroke represents one of the major causes of mortality and permanent disability among the elderly people worldwide. Ischemic stroke results from interrupted blood flow to a portion of the brain that leads to irreversibly damaged ischemic core and a functionally impaired surrounding region of the brain known as the penumbra[1]. Cell death occurs days after initial ischemic insults known as the delayed neuronal death [2]. Loss of integrity of the blood-brain barrier (BBB) resulting from ischemia/reperfusion leads to vasogenic brain edema, a primary cause of stroke-associated mortality [1]. Because most neuroprotective drugs do not cross the BBB, it is not unforeseen that most disorders of the CNS could benefit from improved drug therapy. Moreover, decreased endogenous antioxidant defenses with age may further accelerate ischemic injuries [3]. Nanoparticles (NPs) that have been devised nowadays for drug delivery purposes can readily cross the BBB without compromising its integrity, have enormous applications in the treatment of ischemic stroke [4]. Our group devised a long circulating polymeric nanocapsule made of polylactide-co-glycolide (PLGA) which can encapsulate flavonoids efficiently and can cross the BBB and provide sustained drug release avoiding degradation process. Moreover, PLGA nanoparticles encapsulating flavonoids might play a neuroprotective role by preventing neuronal loss in different vulnerable brain regionsthereby decreasing the mortality rate in ischemic stroke.

## 2. PATHOPHYSIOLOGY OF ISCHEMIA

Ischemic stroke is associated with obstruction of glucose and oxygen transport to the brain causing bioenergetic failure which leads to oxidative stress, inflammation, blood-brain-barrier (BBB) dysfunction, and finally resulting in brain damage [5]. The BBB is formed by a complex system of endothelial cells, astroglia, pericytes, and perivascular mast cells that prevent the passage of most circulating molecules [6]. The tightness of the BBB is ascribed mainly to the vascular layer of brain capillary endothelial cells that are organized side-by-side by tight junctions [7]. These tight junctions prevent the pharmacological therapy of a number of neurological diseases. Studies have shown that cerebral ischemia can cause BBB disruption and increased cerebral vascular permeability, resulting in the formation of brain edema and impaired movements [7]. Motor impairment associated with cerebral stroke often results in short-term or permanent disabilities, substantially impairing patients' capabilities of sensory processing, communication, cognition and motor function [8]. Several brain regions viz. cerebrum, cerebellum, hypothalamus and hippocampus are more susceptible to ischemia than others [5]. A brief period of global brain ischemia causes pyramidal neuronal death in hippocampal CA1 subfield post-reperfusion in rodents and humans [2]. The reason for such phenomenon, commonly referred to as delayed neuronal death, has not yet been fully understood. Discrete populations of hippocampal neurons exhibit differential vulnerability to ischemia leading to difficulties in forming new memories (ante retrograde amnesia), and often affects memories formed before the damage (retrograde amnesia) [9, 10]. Therefore, approaches for protecting

BBB integrity and reducing BBB permeability during cerebral stroke could possibly lead to novel therapies for treating the disorder with greater efficacy.

## 3. ISCHEMIC STROKE IN THE ELDERLY

Cerebral stroke affects mainly the elderly, a population segment that is significantly increasing due to the improved life expectancy. Apart from being a risk factor of stroke, old age has been associated with an increased susceptibility to stroke and poor recovery from brain injury. The effects of ageing on the brain and cognition are extensive, resulting in gross changes in the molecules, cells, vasculature, and overall morphology [11]. The brain shrinks with age, particularly in the frontal cortex. With ageing vasculature and increased blood pressure, the possibility of stroke and ischemia increases with incidences of white matter lesions and memory impairment. Although the fundamental mechanisms are yet to be understood, emerging evidences point towards reactive oxygen species (ROS) as one of the primary determinants of aging [12]. These oxygen radicals are vital contributors to delayed neuronal death (Figure 1). In addition, this highly oxidative stress may damage the mitochondria themselves. Therefore it is essential to have a constant supply of oxygen to maintain normal brain functionality. Mitochondria are the chief source of cellular ROS and contain different enzymes that convert molecular oxygen to superoxide [13] or its derivative hydrogen peroxide (H2O2) (Figure 1). Furthermore, the importance of ROS generation in ischemic cell death derives definitive support from studies demonstrating that antioxidant treatment ameliorates nerve cell injury and death in animal models of stroke [14]. Thus the levels of cellular antioxidant enzymes become significant during the ageing process, since antioxidants are known to check ROS reactions that would otherwise damage macromolecules.

## 4. EFFECT OF ROS GENERATION ON BLOOD BRAIN BARRIER I/R INSULT

Reactive oxygen species (ROS) have been implicated in the pathophysiology of several neurological disorders and brain dysfunctions. Evidence has gathered in the past three decades showing reactive oxygen and nitrogen species mediated oxidative insults can cause cellular damage and death in cerebral ischemia and reperfusion [14]. ROS generation and subsequent oxidative stress result in altered expression and molecular organization of the tight junction proteins leading to increased solute leak [15] across the BBB following ischemic insults. This enables substantial movement of vascular fluid across the microvascular endothelium resulting in vasogenic edema [16, 17, 18, 19]. Oxidative injury becomes worse when blood flow is restored during reperfusion [5]. During reperfusion, mitochondria which play a central role in the development of reperfusion injury use oxygen as a substrate to generate ROS, resulting in an abrupt increase of multiple markers of oxidative damage [14]. ROS contribute to brain injury after I/R insults because the antioxidant defenses of brain are relatively modest. Nitric oxide (NO) which plays an imperative role in the nervous system [19] are produced in large amounts by inducible nitric oxide synthase (iNOS) and contribute to the late stages of cerebral ischemia [20]. Since reperfusion injury is associated with an imbalance of oxidative stress and antioxidant defense system, it is necessary to introduce exogenous antioxidants as drugs as well as free radical scavengers to limit oxidative damage and ameliorate disease progression, especially in aged individuals. Although several natural and synthetic antioxidants have shown neuroprotective effect in ischemic stroke, the anti-I/R agents available are still far from satisfactory. Moreover, the study of such antioxidants is limited by bioavailability and undefined secondary effects when introduced into an in vivo environment.

## 5. PROTECTIVE EFFECTS OF BIOFLAVONOIDS DURING I/R INSULTS

Recently, plant polyphenols have drawn much attention as they exert a variety of biochemical and pharmacological effects because of their antioxidant, anti-inflammatory, and antiproliferative activities [21]. To gain a better insight of the role played by bioflavonoid antioxidants in limiting damage during cerebral ischemic insults in the aged population, a number of studies have focused on the activity of these antioxidants during cellular ageing. Flavonoids are known to check endothelial dysfunctions, atherosclerosis, hypertension and thrombosis [21]. All these mechanisms are linked with the prevention of stroke. Quercetin (QC) is an essential bioflavonoid polyphenolic antioxidant which is found at high concentrations in fruits, vegetables, tea and red wine and is known to exhibit strong free radical scavenger like charateristics. QC results in a diminution in ischemia–reperfusion injury by interfering with inducible nitric oxide synthase activity. Studies have also shown that QC was capable of scavenging superoxide anions released during reperfusion after forebrain ischemia in rat model [22]. However, the mechanistic effects of QC on the cognitive deficits accompanying cerebral ischemia are not fully understood. Moreover, despite being a well known bioflavonoid, QC is unable to cross the BBB due to its hydrophobicity and low oral bioavailability which is a major stumbling block in CNS therapeutics [5]. Therefore, it becomes fundamental to construct a system which could bestow with an elevated pool of such polyphenolic antioxidants in the brain thereby imparting complete protection of neuronal cells against oxidative attack.

## 6. IMPACT OF NANOMEDICINE IN CEREBRAL STROLE

Nanomedicine is certainly one of the most scientific and technological challenges available in the recent years. The size of the nanocapsules which can range between 1 and 300 nm regardless of the type that is used is an essential factor for improved therapeutic abilities. The core to surface ratio of the NPs changes with variation in sizes [23]. Smaller NPs have been

shown to possess a smaller core to surface ratio, which results in immediate release of a drug once the NP's membrane is contravened. Larger NPs therefore are not ideal due to the irregular drug release [23]. Moreover, the flexibility to adapt with other cellular components (i.e., antibodies, peptides) puts the NPs in a favorable position regarding their use as delivery vehicles. Long circulating polymeric NPs made of polylactide-co-glycolide (PLGA) are most conventional efficient drug delivery vehicles because of various technological advantages viz., long shelf life, biodegradability and biocompatibility, protection of drug from degradation, sustained release, high encapsulation efficiency and feasibility of various routes of administration including oral route [24]. Moreover, PLGA NPs have been approved by FDA and European Medicine Agency for parenteral administration, thus reducing the time for human clinical applications. These drug delivery systems therefore, proposed to be an intriguing tool that has a potential ability to solve the unmet problem of enhancing drug transport across the BBB (Figure 2). The likelihood for BBB-impermeant drugs to reach the brain, when carried by NPs, is based upon the fact that they cross the barrier depending completely on the physicochemical features of the NPs vehicle and are independent of the chemical structure of the drug, which is encapsulated inside the NPs [25]. Biodegradable nanoparticles formulated from poly (D,L-lactide-co-glycolide) (PLGA) have been extensively investigated for sustained and targeted delivery of different agents, including recombinant proteins, plasmid DNA, and low molecular weight compounds [26]. PLGA NPs present some very attractive properties such as biodegradability and biocompatibility, protection of drug from degradation, possibility of sustained release, and the possibility to modify surface properties to target nanoparticles to specific organs or cells [24].

In a study conducted by us, we tried to enumerate whether treatment with PLGA nanoparticulated quercetin via oral gavage put forth any neuroprotective effect against cerebral-ischemia reperfusion evoked neuronal damage in different brain regions of young and aged rats.

## 7. FORMULATION OF NANOENCAPSULATED QUERCETIN

Emulsion-diffusion-evaporation is one of the most frequently used methods for the preparation of nanoparticles (Figure 3a). The preparation steps involve emulsification of the polymer solution into an aqueous phase followed by polymer solvent evaporation, inducing polymer precipitation as nanospheres. The nanoparticles are then collected by ultracentrifugation and washed well with distilled water to remove any unencapsulated drug and lyophilized for storage [27]. A modified methodology of emulsion – diffusion – evaporation [5] was used to formulate PLGA- nanoencapsulated QC. Briefly, 36 mg of PLGA and 2.7 mg of QC were dissolved in ethyl acetate at room temperature. The organic solution of PLGA and drug in ethyl acetate was then added dropwise to 5 ml of an aqueous phase containing DMAB. The organic/water emulsion was then stirred at room temperature for 3 h followed by homogenization. The organic solvent was evaporated by constant stirring and the aqueous suspension of nanoencapsulated QC was ultracentrifuged at 105,000 g for 1 h. The nanocapsule pellet thus obtained was washed with phosphate-buffered saline (PBS) twice and resuspended in 2 ml PBS along with sucrose and glucose (used as a cryoprotectant) added at a concentration of 20%. The nanosuspension was lyophilized and preserved at -20°C for future use.

**7.1. Characterization of nanoparticles encapsulated QC using Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM).** Transmission electron microscopy (TEM) has been known to be the first method used to find out the size and size distribution of nanoparticle samples [28]. First the images of the nanoparticle samples are obtained followed by counting of particles, ideally a few thousand, in order to achieve a good statistics on the size and size distribution of the nanoparticles. For this computer image processing software are of utmost interest for counting a moderately large number of particles with greater precision. In our study, TEM analysis was done with nanoencapsulated QC suspension being placed primarily upon 300 mesh carbon coated copper grids followed by staining with 2% Phosphotungstic acid solution and vigorous washing. The stained grids were then dried again, placed in the sample receiver and the images of the samples thus obtained showed particle diameter using the TEM Software program at different magnifications. The atomic force microscopy (AFM) analysis was performed with a picoview 1.10.4 version AFM system (Agilent Technologies, USA). Mica was selected as a solid substrate and used immediately after cleavage in a clean atmosphere. The nanoencapsulated QC suspensions on mica were dried in air (65% humidity) for 30 min and the images were analyzed with the help of Pico Image Software from Agilent Technologies, USA. The nanocapsules obtained were found to be in the size range 20–50 nm. Observations made from TEM and AFM images (Figure 3b-c) revealed that nanocapsules were spherical in shape with a narrow size distribution. Encapsulation efficiency of the quercetin nanoparticles was found out to be about 80%.

## 8. INDUCTION OF CEREBRAL ISCHEMIA-REPERFUSION

Ischemic brain damage in the rat via surgical induction is an extensively used model for stroke research. Here we demonstrate the induction of cerebral ischemia via bilateral clamping of the common carotid arteries. Male Sprague Dawley rats of both age groups were anesthetized and made ischemic. Following 30 min. of ischemic insult, animals were subdivided into three groups (both young and aged) subjected to reperfusion (by withdrawing clamps) intervals of 30 min, 24 h and 72 h respectively. Postoperative care was properly taken in animals that underwent reperfusion for 24h and 72 h till day 3. Post-reperfusion, following sacrifice by decapitation, different brain regions viz. hippocampus, hypothalamus, cerebral cortex and cerebellum were isolated. Small portions of the brain regions were stored for the analysis of edema development. Rest of the isolated brain regions were then homogenized in phosphate buffer saline with Teflon coated homogenizer for different biochemical assays. A part of the hippocampal region was isolated from the brain tissue of animals that underwent reperfusion for 24 and 72 h for in order to account for the effect of nanoencapsulated quercetin on delayed neuronal death due to I/R insults.

**8.1. Effect of nanoencapsulated quercetin on ROS generation and mitochondrial membrane microviscosity in I/R induced rats.**

Mitochondria play a vital role in reperfusion injury via excessive reactive oxygen species (ROS) production thereby damaging cellular components, and initiating cell death. These overproduced ROS result in macromolecular damage and activation of various pathways. Evidences have shown that mitochondria play a fundamental role in energy metabolism. As a result, generation of reactive oxidative species (ROS), and regulation of the cell death pathway undergo severe damage during ischemic injury [29, 30]. Mitochondrial dysfunction in cerebral ischemia resulting from impaired delivery of glucose and oxygen to the brain tissue [31, 32] is abruptly caused by damage to the mitochondrial energy metabolism. The mitochondrial membrane potential (MMP) provides a driving force triggering ATP synthase thereby generating high-energy phosphate. MMP was disturbed during cerebral ischemia. Studies have suggested that extensive loss of MMP was considered to be a common feature of ischemic injurious processes that favour the opening of mPTP as well as initiation of the apoptotic cascade [33, 34]. Moreover, studies indicated that mitochondrial ROS production played an essential role in reperfusion injury following I/R insults [35]. Occurrence of MPT was found to be closely related to oxidative stress promoting ischemic neuronal death [36]. In this study, the generated ROS were assessed with the membrane-permeable fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). Higher level of ROS was observed to be increased in all the brain regions of normal aged animals (Table 1) [5].

**Table 1.** Effect of nanocapsulated quercetin on ROS generation (DCF-Fluorescence) in different brain regions of ischemia-repurfusion induced young and aged rats.

| Experimental Conditions | DCF-Fluorescence (% of normal) of different brain regions of young and aged rats | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Young rats | | | | Aged rats | | | |
| | Cerebral Cortex | Cerebe-llum | Hypoth-alamus | Hippoca mpus | Cerebral cortex | Cerebellum | Hypothalamus | Hippocampus |
| **Normal** | 101.86 ± 0.844 | 101.65 ± 0.831 | 103.81 ± 0.836 | 102.97 ± 0.841 | 109.88 ± 0.894 | 106.67 ± 0.671 | 107.83 ± 0.618 | 115.88 ± 0.969 |
| **Cerebral ischemia repurfused (A)** | 431.77 ± 0.849* | 433.56 ± 0.845* | 445.67 ± 0.837* | 478.33 ± 0.869 | 502.79 ± 0.832* | 504.35 ± 0.551* | 507.89 ± 0.771* | 531.72 ± 0.912* |
| **(A)+Free QC treated** | 427.54 ± 0.824 | 429.43 ± 0.681 | 440.79 ± 0.857 | 466.44 ± 0.776 | 501.46 ± 0.885 | 504.31 ± 0.551 | 505.92 ± 0.662 | 514.36 ± 0.763 |
| **(A)+Nano capsulated QC treated** | 110.56 ± 0.808** | 108.66 ± 0.809** | 108.71 ± 0.809** | 112.82 ± 0.875** | 113.38 ± 0.823** | 111.24 ± 0.859** | 114.56 ± 0.860** | 125.92 ± 0.979** |

Values are mean ± SE of rats. *,P<0.001 significantly different from normal; **, P<0.001 significantly different from ischemia-repurfused rats treated.

**Table 2.** Effect of NPQC treatment on the mitochondrial membrane microviscosity ([r0/r − 1]− 1) of different brain regions of Ischemia-Repurfusion induced aged and young rats.

| Experimental Conditions | Membrane Microviscosity ($[r_0/r - 1]^{-1}$) of Rat Brain regions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Young rats | | | | Aged rats | | | |
| | Cerebral Cortex | Cerebe -llum | Hypoth-alamus | Hippocam pus | Cerebral Cortex | Cere-bellum | Hypothala mus | Hippoca mpus |
| **Normal** | 0.571 ± 0.0008 | 0.569 ± 0.0007 | 0.575 ± 0.0008 | 0.573 ± 0.0008 | 0.542 ± 0.0007 | 0.539 ± 0.0007 | 0.547 ± 0.0007 | 0.526 ± 0.0007 |
| **Cerebral ischemia repurfused (A)** | 0.261 ± 0.0007* | 0.265 ± 0.0008* | 0.270 ± 0.0008* | 0.223 ± 0.0007* | 0.198 ± 0.0009* | 0.191 ± 0.0009* | 0.201 ± 0.0008* | 0.162 ± 0.0009* |
| **(A)+Free QC treated** | 0.263 ± 0.0007 | 0.266 ± 0.0008 | 0.274 ± 0.0008 | 0.224 ± 0.0007 | 0.203 ± 0.0009 | 0.195 ± 0.0009 | 0.205 ± 0.0008 | 0.162 ± 0.0009 |
| **(A)+Nano capsulated QC treated** | 0.569 ± 0.0008** | 0.565 ± 0.0007** | 0.572 ± 0.0008** | 0.562 ± 0.0007** | 0.530 ± 0.0007** | 0.531 ± 0.0007** | 0.538 ± 0.0008** | 0.514 ± 0.0007** |

In young rats, short term ischemia (30 min) followed by 30 min of reperfusion resulted in considerable increase in ROS level in different brain regions, the highest level being observed in the hippocampus. Pre-treatment with free quercetin before ischemic induction, resulted in almost no significant protection against I/R mediated ROS generation in brain regions in both age

groups of animals. The fluidity of mitochondrial membranes is especially crucial for energy production. In the present study, mitochondrial membrane microviscosity in different brain regions of both age groups of rats was found to be reduced post cerebral ischemic insult (Table 2) [5], the worst affected area being the hippocampus. Free quercetin treatment resulted in no significant improvement in mitochondrial membrane microviscosity, whereas nanocapsulated qu ercetin treatment imparted complete protection to the mitochondrial membrane of all the brain regions in both young and aged rats (Table 2).

**8.2. Effect of nanocapsulated QC on antioxidant defences in I/R induced rats.**

The antioxidant enzyme capacity of the brain tissue is supposed to be among the major mechanisms by which cells neutralize the lethal effects of ROS after ischemic injury. Overproduction of ROS results in oxidative stress that leads to shift in the balance between oxidant/antioxidant statuses. This eventually leads to potential cellular damage, thereby contributing in the initiation of neuronal damage in global cerebral ischemia. The endogenous antioxidant defense against ROS involves the joint action of three main intracellular antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) that are present in cytosol and peroxisomes, respectively [37]. Ischemia reperfusion induced age related alteration in different antioxidant enzymes viz. superoxide dismutase (SOD) and catalase in different brain regions is shown in Table 3 [5].

**Table 3.** Effect of Nanocapsulated Quercetin on the decreased antioxidant enzyme activities in young and aged rat brain regions by the induction of cerebral ischemia and repurfusion.

| Experi -mental Condition | Superoxide dismutase ( percentile autoxidation of pyrogallol) | | | | | | | | Catalase (µmol/mg protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Young rats | | | | Aged rats | | | | Young rats | | | | Aged rats | | | |
| | Cerebral Cortex | Cerebe -llum | Hypoth -alamus | Hippoc ampus | Cerebral cortex | Cere- Bellum | Hypot halamus | Hippoc ampus | Cerebral Cortex | Cerebe llum | Hypothal amus | Hippoc ampus | Cerebral -cortex | Cerebe -llum | Hypothal -amus | Hippoca mpus |
| Normal | 50.69 ± 3.925 | 63.94 ± 0.827 | 50.94 ± 1.497 | 66.16 ± 1.024 | 26.05 ± 1.213 | 23.92 ± 0.706 | 24.74 ± 0.477 | 18.7 ± 0.204 | 0.421 ± 0.004 | 0.442 ± 0.006 | 0.441 ± 0.005 | 0.418 ± 0.003 | 0.261 ± 0.004 | 0.270 ± 0.005 | 0.257 ± 0.004 | 0.212 ± 0.003 |
| Cerebral ischemia repurfused (A) | 26.83 ± 1.633 | 22.38 ± 1.312 | 19.37 ± 0.770 | 17.48 ± 0.714 | 8.36 ± 0.362 | 12.15 ± 0.392 | 14.26 ± 0.425 | 9.37 ± 0.090 | 0.240 ± 0.006 | 0.239 ± 0.004 | 0.189 ± 0.006 | 0.191 ± 0.006 | 0.163 ± 0.007 | 0.168 ± 0.004 | 0.171 ± 0.005 | 0.153 ± 0.006 |
| (A)+free QC treated | 28.17 ± 1.454 | 21.75 ± 1.427 | 20.94 ± 0.520 | 18.76 ± 0.630 | 11.45 ± 1.281 | 13.44 ± 0.142 | 15.15 ± 0.454 | 11.65 ± 1.340 | 0.273 ± 0.007 | 0.250 ± 0.004 | 0.260 ± 0.004 | 0.199 ± 0.004 | 0.179 ± 0.003 | 0.204 ± 0.004 | 0.176 ± 0.003 | 0.153 ± 0.006 |
| (A)+Nanocapsu -lated QC treated | 49.39 ± 31.68 | 49.75 ± 1.544 | 50.54 ± 0.912 | 51.84 ± 1.440 | 20.21 ± 0.579 | 22.31 ± 0.375 | 22.18 ± 1.377 | 13.19 ± 0.358 | 0.388 ± 0.004 | 0.444 ± 0.008 | 0.381 ± 0.006 | 0.358 ± 0.005 | 0.222 ± 0.003 | 0.228 ± 0.006 | 0.189 ± 0.005 | 0.178 ± 0.004 |

Values are expressed as mean± S.E of rats.

Both SOD and catalase activities were observed to be significantly reduced in all the brain regions, especially the hippocampal region of normal aged rats when compared to that of normal young rats. Ischemia-reperfusion induced a further reduction both in SOD and catalase activities in the aged variety mostly in the cortex and hippocampal regions when compared to those of the young group of animals. Free quercetin treatment prior to ischemic insult resulted in no significant protection of those endogenous antioxidants. Treatment with nanoencapsulated quercetin induced complete protection to these enzymes against any degradation of these enzymes from ischemic insult. Glutathione is the most important intracellular antioxidant and upon I/R insults, was found to be significantly decreased in the brain regions of both age groups of rats, the most affected region being the hippocampus (Figure 4). The cellular GSH content was significantly augmented in both groups of young and aged rats that received nanoparticulated quercetin (2.7 mg/kg. b. wt.) prior to ischemic insult, whereas free quercetin hardly showed any significant effect in the brain regions of both young and aged group of rats subjected to ischemia reperfusion (Figure 4).

**8.3. Effect of nanocapsulated quercetin on edema of the brain in I/R induced rats.** Brain edema is a well known critical impediment of cerebral infarction. Ischemic brain edema occurs due to cell swelling also known as cytotoxic edema and increased permeability of the blood vessels known as vasogenic edema. A change in brain tissue osmolality is the index of cerebral edema. Cerebral edema

results in decreased cerebral tissue osmolality post I/R insults (Figure 5). Treatment of ischemia induced rats with nanoencapsulated quercetin resulted in an increase in brain tissue osmolality whereas free quercetin treatment showed no significant improvement.

**8.4. Effect of nanoencapsulated quercetin on hippocampal iNOS and caspase-3 protein expressions in I/R induced rats.** Nitric oxide (NO) is a free radical with signaling functions in the CNS. iNOS is the inducible form of nitric oxide synthase (NOS) that is expressed in different cells in response to various proinflammatory stimuli [38]. In the nervous system, iNOS generating NO has both neuroprotective and neurotoxic effects [39]. In our study we found out that the hippocampus was the worst affected amongst all other brain regions of rats exposed to I/R insult. Further studies were therefore conducted on hippocampal region of both age groups of rats. Earlier studies have shown that there are three isoforms of the enzyme, nitric oxide synthase (NOS) namely neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), which generate nitric oxide (NO) via the combination of L-arginine and molecular oxygen [40, 41]. Studies have shown that inducible nitric oxide synthase (iNOS) produces toxic levels of nitric oxide (NO) and is expressed in various brain pathologies, including cerebral ischemia. It has been established that iNOS is normally expressed at early stage of reperfusion after brain ischemia [42, 43]. NO acts as a reactive nitrogen species and is capable of inducing necrotic and apoptotic cell death which again depends on the intensity of toxic insults of NO (Bonfoco et al., 1995). During experimental cerebral ischemic attacks, overproduction of NO due to iNOS can interact with superoxide anion, resulting in the formation of the powerful oxidant species peroxynitrite. NO and peroxynitrite may stimulate caspase-dependent cell death due to endoplasmic reticulum stress. Caspases are cysteine proteases that play a fundamental role in apoptotic cell death [44]. Caspase-3, an executioner caspase, plays an essential role in apoptosis in a wide variety of cells. Activated caspase-3 has been shown to cleave proteins that are essential in the maintenance of neuronal process integrity. Neurons are the major cell population that undergoes caspase-3–dependent apoptosis, beginning several hours after hypoxia-ischemia. Western blot analysis indicated an upregulated expression of iNOS in the hippocampus of rats (Figure 6) that were exposed to I/R insults for 30 min, 24 h and 72 h. Free quercetin treatment showed no significant variations in iNOS expression whereas treatment with nanoencapsulated quercetin resulted in significant downregulation of iNOS expression in the hippocampal region of both age groups of rats (Figure 6). Ischemic insult followed by various reperfusion intervals resulted in an increased caspase-3 activity in the hippocampal region of both age groups of rats (Figure 7). Free quercetin treatment showed no significant improvement whereas nanoencapsulated treatment imparted significant protection via minimizing the caspase-3 activity in the hippocampal region of both young and aged rats (Figure 7).

**8.5. Effect of nanoencapsulated quercetin on pyramidal nerve cell count of the hippocampus of I/R exposed rats.** The mechanism of learning and memory is known to be closely associated with the hippocampal region of the brain. It is the most frequently examined region during the mechanistic studies of ischemic cell death and selective neuronal vulnerability. Studies have shown that a brief period of ischemic insult caused hippocampal cell death in the CA1 and CA3 pyramidal neurons [2]. Ischemia-related cell death includes mechanisms viz., necrosis, apoptosis and autophagic forms of cell death [45]. Cellular necrosis begins with the end of normal physiology and gradually commencing towards ATP depletion and impairments in membrane permeability followed by nuclear pyknosis [45]. As a result, structural integrity of the organelles gets affected. Apoptosis is characterized by changes in gene expression and protein activity. It begins with chromatin condensation followed by nucleolar disintegration resulting in the formation of fragmented dying cells, also known as apoptotic bodies [47]. In our study, analysis of the data obtained from hippocampal nerve cell count of control animals of both age groups (Figure 8) revealed that there ageing has got a significant effect on pyramidal neuron density in both CA1 and CA3 subfields of the hippocampus. Ischemic insults followed by various reperfusion intervals resulted in a significant decline in the neuronal density in the hippocampal subfields of both young and aged rats. Neuronal loss was also evident in the other two hippocampal regions viz. dentate gyrus and hilus of both age groups of rats. Free quercetin treatment produced almost no improvement in the nerve cell count whereas nanoencapsulated quercetin treatment proved to be highly efficient restoring normal neuronal counts with long lasting effects even post operation in both aged and young rats (Figure 8).

## 9. SUMMARY AND CONCLUSION

Till date various neuroprotective agents used in preclinical studies have shown potential but they have failed at clinical trials because of either safety issues or lack of efficacy. Restriction imposed by BBB is yet another important factor that hampers the

delivery of many potentially therapeutic neuroprotectants and diagnostic compounds to the brain. Moreover, enhanced production of ROS and subsequent oxidative stress has been thought to play a key role in cerebral ischemia reperfusion especially in the elderly where evidences of neuronal degeneration arising from oxidative stress are very common. Mitochondria are the main resource of ROS [48]. The process of oxidative phosphorylation involves oxidation of NADH to produce energy in the inner mitochondrial membrane resulting in ROS generation [49]. In the human body, cells under aerobic condition are always susceptible to the insult of ROS. However the antioxidant enzyme status of the brain is predominantly significant for the primary endogenous defense against ROS induced injury and involves a combined effect of the intracellular antioxidant enzymes such as SOD and catalase. Reduced glutathione (GSH) is known to play a critical role in antioxidative defenses via regeneration of antioxidants and by reduction of hydroperoxide via the glutathione peroxidase cycle.

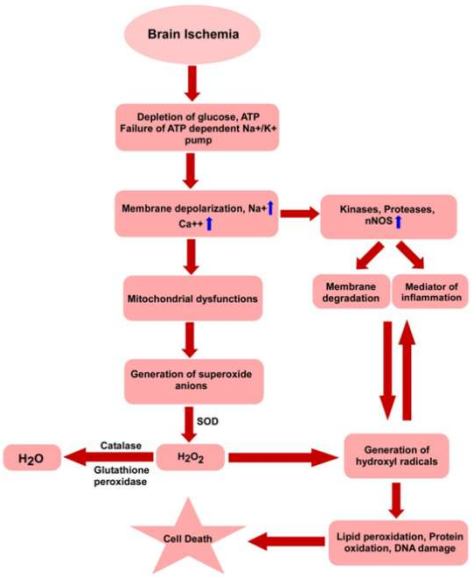


**Figure 1.** Schematic preparation of nanoencapsulated quercetin. b. TEM pictures of nanoencapsulated quercetin at a magnification of 20,500X demonstrating spherical particles with an average size distribution from 10–50 nm. c. AFM images of nanocapsules. Amplitude-flattened view of nanoparticlulated quercetin is shown.

Whenever the equilibrium between ROS production and antioxidant defense is lost, oxidative stress develops which through a series of events destroy the normal cellular functions leading to various pathological conditions (Figure 1).

There is a gradual decline in the normal antioxidant defense mechanisms in the aging brain, as well as in the case of several neurodegenerative diseases, where the vulnerability of the brain to the deleterious effects of oxidative damage increases [50]. Applications of nanotechnology for neuroprotection against ischemic injuries have focused on restricting the detrimental effects of these free radicals generated after injury. Previous studies have shown that during cerebral stroke, the BBB is severely compromised and its normal functions get disrupted for as long as 7 days post-trauma [51] leading to further neuronal damage. Therefore, treatment should be made target-specific where the administered drugs will be able to protect their molecular structure and therapeutic abilities. Nanoparticles (NPs), which can readily circumvent the BBB (Figure 2) without compromising its integrity, have enormous applications in the therapeutic intervention of ischemic stroke. The size and size distribution of nanoparticles are essential in determining their stability and cellular uptake efficiency [52].

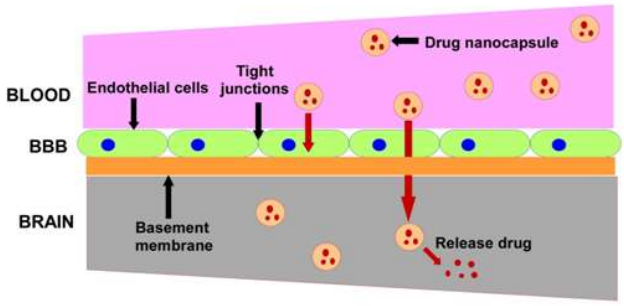


**Figure 2.** Oxidative stress in ischemic brain damage leads to neuronal cell death via caspase and nNOS dependant signaling mechanisms.

PLGA nanoparticles encapsulated bioflavonoid antioxidant, quercetin was shown to have positive outcome in treating ischemia-reperfusion mediated neuronal damage in rats (Ghosh et al., 2013).

Nanoparticles that are less than 100 nm in size (Figure 3) [5] have a longer circulation time in the blood and experience reduced hepatic filtration with no aggregation that made the delivery system very much appropriate for drug delivery to target organs, especially the brain, which had always remained the critical challenge in drug delivery.

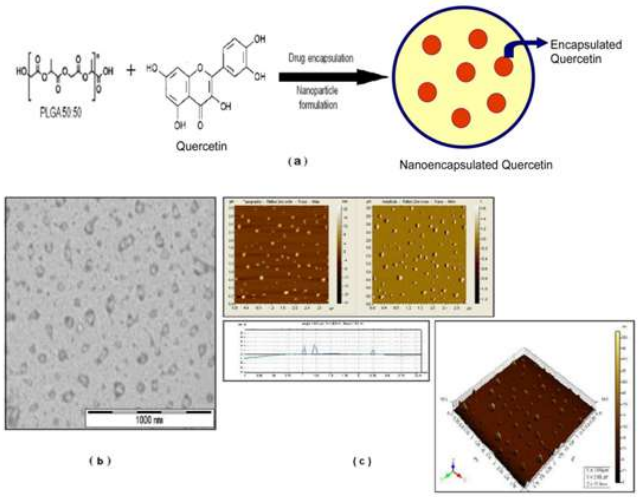


**Figure 3.** Drug encapsulated nanoparticles cross the blood brain barrier via the endothelial tight junctions.

Nanoparticulated quercetin treatment prior to I/R insult in rats showed promising results by imparting absolute protection to enzymatic antioxidant systems (Figure 4 and Tables 3) [5]whereas free quercetin showed no level of improvement.

Ischemia depletes brain cells of energy substrates thereby resulting in failure of cell membrane ionic pumps, leading to brain edema. Progression of edema resulted in reduction of neuronal osmolality and loss of blood-brain- barrier integrity [53]. Treatment with nanoencapsulated quercetin prior to ischemic insult restricted osmolality in different brain regions of both groups of rats (Figure 5) [5]. ROS generation was also minimized thereby imparting protection against ROS induced oxidative damage (Table 1). The decreased ROS therefore becomes capable

of establishing a balance between the increased lipid peroxidation and depleted glutathione and antioxidant enzyme levels.

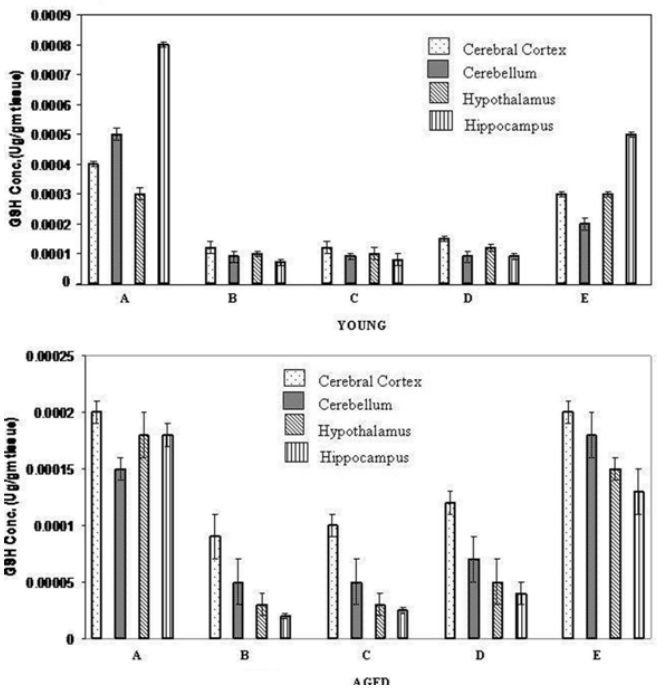


**Figure 4.** Effect of quercetin nanoparticles on GSH concentration in different brain regions of young and aged rats exposed to ischemic insult. A. Normal, B. Ischemia-Reperfusion induced, C. B+Free QC treated, D. B+ Empty nanoparticle treated, E. B+ Nano QC treated. Values are mean ± SE of rats.

Moreover, the maintenance of mitochondrial membrane fluidity of the brain cells was shown to be achieved by the defensive actions of nanoparticulated quercetin where as a significant drop in mitochondrial membrane microviscosity occurred in different brain regions of rats irrespective of age due to the induction of I/R insults (Table 2).

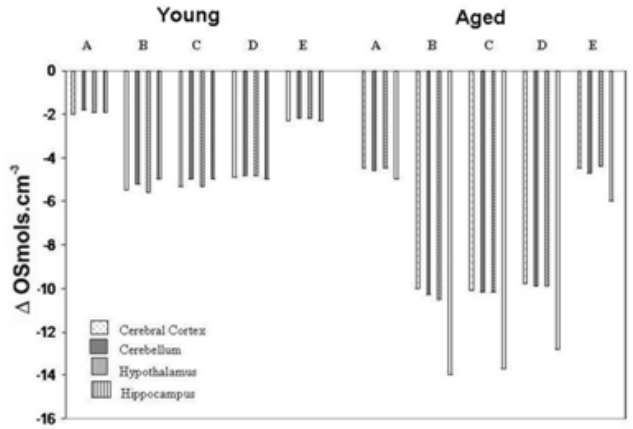


**Figure 5.** Effect of nanoencapsulated quercetin on cerebral edema in different brain regions of I/R induced young and aged rats. A. Normal, B. Ischemia Reperfusion induced, C. B+ Free QC treated, D. B+ Empty nanoparticle treated, E. B+ Nano QC treated Values are mean ± SE of rats.

Inhibition of release of iNOS yet again might have a significant implication in understanding the novel therapeutic strategy targeted specifically against the progression of ischemic brain injury. Expression of iNOS in the hippocampal region of ischemia-reperfusion induced rats reached its highest peak after 72 h of reperfusion (Figure 6) [5]. Highest protection against I/R induced iNOS expression in the hippocampal brain region was shown to be imparted in those groups of animals that were orally treated with nanoencapsulated quercetin prior to ischemic insults as well as post-operation (Figure 6).

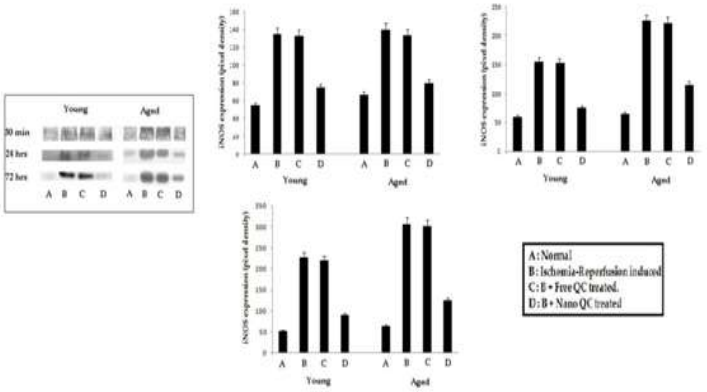


**Figure 6.** Western blot analysis to show the effect of quercetin nanoparticles on the hippocampal iNOS release of young and aged rats subjected to ischemia followed by reperfusion for 30 min, 24 h and 72 h. A. Normal, B. Ischemia-Reperfusion induced, C. B+ Free QC treated, D. B+ Nano QC treated. Values are mean ± SE of rats.

Ischemic insults resulted in delayed neuronal death in selectively vulnerable hippocampal subfields. Cerebral ischemia activates two general apoptotic pathways: the intrinsic pathway, which originates from mitochondrial release of cytochrome c and related stimulation of caspase-3; and the extrinsic pathway that originates from the activation of cell surface death receptors, thereby resulting in the stimulation of caspase-8. Caspase 3 has been identified as a potent mediator of apoptosis [54]. Prolonged periods of reperfusion after 72 h resulted in remarkable increase in hippocampal caspase 3 activities (Figure 7) [5] prior to ischemia and post reperfusion.

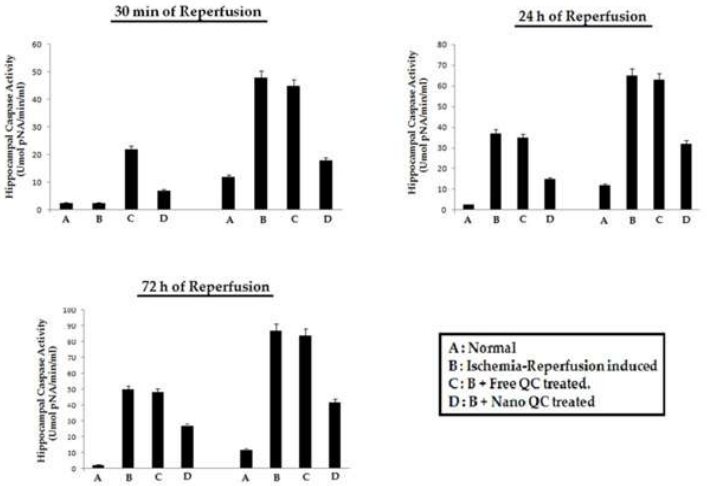


**Figure 7.** Effect of nanoencapsulated quercetin on hippocampal caspase-3 activity of ischemia-reperfusion induced young and aged rats. A. Normal, B. Ischemia-Reperfusion induced, C. B+ Free QC treated, D. B+ Nano QC treated. Values are mean ± SE of rats.

However, treatment with nanoencapsulated quercetin restricted the increased activity of caspase-3 in the hippocampal region of both age groups of rats, thereby controlling the apoptotic

processes, leading to hippocampal neuronal cell death. Moreover, a significant reduction in the pyramidal neuronal density in the CA1 and CA3 hippocampal subfields of I/R induced animals (Figure 8) [5] was observed. The severity of neuronal loss in the hippocampal subfields was observed to be maximum upon increasing the reperfusion intervals. However, the damage was to some extent curbed following oral treatment with nanoencapsulated querctin (Figure 6). Mortality rate was also controlled with majority of the post-operative death that took place following 3 days of I/R insults. 50% mortality among the aged animals was reported after 24 h of reperfusion. However, nanoencapsulated querectin treated animals, irrespective of both age groups, survived till day 3 following reperfusion.

From the ongoing discussions we understand that stroke therapy is restricted by many limitations and its therapeutic intervention is significantly small. An effective therapeutic agent should be able to provide neurological protection by targeting the BBB route in order to increase the former's efficiency as a neuroprotective agent in the brain. Further progression of effectiveness can be achieved by improved retention of the antioxidant agent at the injury site of the brain.

Treatment with nanoencapsulated quercetin might prove to be effective against I/R induced oxidative damage by restricting the loss of pyramidal neurons from the hippocampal subfields. However, shorter durations of reperfusion pose significantly less damage than longer durations and early treatment with nanodrugs might effectively decrease the mortality rate via reduced neuronal damage. This approach of utilizing a polymeric drug delivery vehicle with an aim to deliver a non-toxic polyphenolic antioxidant, quercetin, to brain offers a potential clinical application in the therapeutic intervention of neurodegenerative diseases in future.

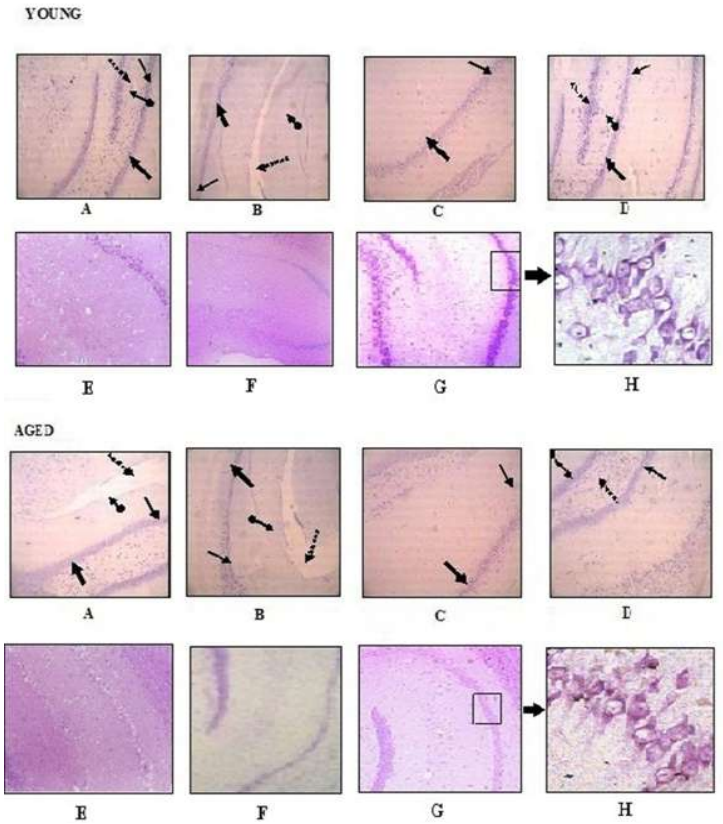


**Figure 8.** Cresyl Violet stained hippocampal cross sections of young and aged rats demonstrating neuronal morphology. A. Normal, B. Ischemia-Reperfusion (30 min) induced, C. B + Free QC treated, D. B+ Nano QC treated. E. Ischemia- Reperfusion (72 h) induced. F. E + Free QC treated; G. E + Nano QC treated, CA1 subfield;CA3 subfield; Dentate Gyrus; Hilus. A. Normal, B. Ischemia- Reperfusion induced, C. B + Free QC treated, D. B+ Nano QC treated.

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