Chapter III

Rutin protects cognitive impairment, oxidative stress and neuroinflammation in intracerebroventricular-streptozotocin injected rats
Introduction:

Alzheimer’s disease (AD) is the progressive neurodegenerative disorder, characterized by impairment of memory and cognitive function. Neuropathological features of AD brain are extracellular accumulation of amyloid beta (Aβ) peptide in the form of plaques, and intracellular deposits of hyper phosphorylated tau proteins (Selkoe, 2001). Several mechanisms of neuronal degeneration in AD have been proposed, such as free radicals generation, oxidative stress, mitochondrial dysfunction, inflammatory processes, genetic factors, environmental factors, apoptosis etc. These factors may interact to each other in a vicious cycle of toxicity leading to neuronal dysfunction, and consequently leads to neuronal cell death. Reactive oxygen species and inflammation factors contribute to the pathogenesis of oxidative stress. Oxidative stress in brain is a condition of imbalance between oxidants and antioxidants. This imbalance can lead to cellular dysfunction by increasing the reactive oxygen species and reactive nitrogen species. The brain is susceptible to lipid peroxidation due to its high oxygen content, low level of antioxidant protection, and high level of polyunsaturated fatty acids, high level of iron and ascorbate (Moreira et al., 2005). It is well documented that free radical induced oxidative damage, particularly to lipids (Butterfield et al., 2001), nucleic acids (Wang et al., 2005) and proteins (Sultana and Butterfield, 2009) in the brain of AD patients. Neuroinflammation is considered to be the downstream consequence of amyloid beta aggregation, which will bring the activation of microglia, initiating the pro-inflammatory cascade that leads to the release of cytokines, chemokines, reactive oxygen species, reactive nitrogen species and various proteolytic enzymes that will result in the degenerative changes of neuron (Eikelenboom et al., 2006).

Cyclooxygenase-1 (COX-1) expression is constitutive in most of the tissues while cyclooxygenase-2 (COX-2) is expressed in response to injury (Mitchell et al., 1993). However, COX-2 is expressed in neuronal cells during the physiological and pathological process. In early stage of AD, COX-2 protein and mRNA are highly expressed in the cortex and hippocampus. In neurodegenerative disorders including Alzheimer’s disease, glial cell activation, neuroinflammation, neuronal cell death and impaired oxidative metabolism are common (Gibson and Zhang, 2002). Glial filament proteins of astroglial cells are highly expressed following injury as evidenced by the in vivo and in vitro studies (Norton, et al., 1992). It is well documented that astrocytes maintain the brain homeostasis (Hertz and Dienel, 2002), and protects the neurons from the oxidative stress by releasing the antioxidant enzymes (Stone et al., 1999). Nuclear transcription factor NF-kB play important role in the
pathogenesis of oxidative stress associated neurodegenerative diseases. NF-kB is one of the most important transcriptional regulators of proinflammatory gene expression. Synthesis of cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, is mediated by NF-kB. Free radical induced damage of DNA single and double strand breaks activates the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1), which plays an important role in gene transcription and defence against oxidative stress (Adamczyk et al., 2005). Over-activation of PARP-1, may lead to cell death due to its rapid utilization of cellular energy sources like NAD⁺ and ATP (Szabò et al., 2006).

Streptozotocin (STZ), a glucosamine-nitrosourea compound, generates a cytotoxic product that preferentially destroys β cells in pancreatic islet and produces diabetes mellitus when it is metabolized. It has been found that intracerebroventricular (ICV) injection of STZ in rats, produces long-term and progressive learning and memory deficits in rats, as well as impaired cerebral glucose and energy metabolism, oxidative stress, neuroinflammation and other biochemical changes that resemble those found in the brain of sporadic Alzheimer's disease (sAD) patients (Lannert and Hoyer, 1998; Salkovic-Petrisic, 2008; Prickaerts et al., 1999; Sharma and Gupta, 2002). It is also reported that ICV-STZ treated rats showed higher expression of hyperphosphorylated tau protein in the hippocampus, and some reports suggested β-amyloid accumulation in the meningeal capillaries were observed, indicating the possibility of developing sAD pathology in this experimental model, that further supporting the resemblance of this experimental model to human sAD (Salkovic-Petrisic et al., 2006; Grünblatt et al., 2007). Nowadays, it is a well established model to study the sporadic dementia of Alzheimer's type.

Rutin is reported as primary flavonoids in a number of plants (Kim et al., 2005). Rutin is used by animal feed, cosmetics, and chemical industries as a natural pigment, stabilizer, food preservative, and UV absorbent (Pu et al., 2005). Rutin has several pharmacological properties such as antioxidant, anti-inflammatory, antiallergic, antiviral, anticarcinogenic and potent scavenger of superoxide radicals (Kamalakkannan and Prince, 2006; Bishnoi et al., 2007). It is reported that rutin supplementation from natural food sources, such as soba noodles or groats, might improved memory impairment and decreased hippocampal pyramidal neurons death such as in Alzheimer's disease. Rutin has the ability to suppress the microglial activation and proinflammatory cytokines (Koda et al., 2009). Rutin has significant neuroprotective effect against lipid peroxidation and increased antioxidant
enzymes in middle cerebral artery occlusion (MCAO) model of cerebral ischemia (Khan et al., 2009).

The aim of the present study was to investigate the pre-treatment effects of rutin on cognitive dysfunction, oxidative stress and inflammatory markers in hippocampus in ICV-STZ model of rat.

**Materials and methods:** As described in section III.

**Results:**

**Behavioural observation**

**Effect of rutin on performance in Morris water maze task:**

**Latency:**

The difference in escape latency was observed onto a hidden platform produced by training session. The latencies to reach platform were decreased gradually in all groups during 5 days of training period in Morris water maze test. The mean latency was increased significantly (p<0.001) in L group as compared to S group, showing a poor learning performance due to ICV-STZ infusion. This disrupted performance of L group was significantly (p<0.001) decreased by the pre-treatment with rutin in R+L group animals (Fig.1).

**Path Length:**

Acquisition performance was improved in all groups of animals in Morris water maze test. S and R+S groups show decreased path length to find the platform from day 2 to day 5 of the experiment. However, L group animals showed a significantly (p<0.001) higher path length than S group animals to find the platform, but R+L group animals has shown a significant (p<0.001) improvement as compared to L group animals (Fig.2).
Fig. 1: Effects of rutin supplementation on escape latency to find the platform in Morris water maze test in ICV-STZ infused rats. Values are expressed as mean±S.E.M. (n=10). Swimming times of four trials per day for 5 days to each group animals are shown. Average escape latency to find submerged platform was significantly (**p<0.001) prolonged in the L group animals when compared to the S group animals. Pre-treatment with rutin has decreased it significantly (###p<0.001) in R+L group animals as compared with the L group animals.

Fig. 2: Effects of rutin supplementation on path length to find the platform in Morris water maze test in ICV-STZ infused rats. Values are expressed as mean±S.E.M. (n=10). Swimming times of four trials per day for 5 days to each group animals are shown. Average distance travelled to find submerged platform was significantly (**p<0.001) prolonged in the L group animals when compared to the S group animals. Pre-treatment with rutin has decreased it significantly (###p<0.001) in R+L group animals as compared with the L group animals.
Effect of rutin on TBARS:
The effect of rutin on TBARS content was estimated to prove the oxidative insult on lipid per oxidation in hippocampus of ICV-STZ infused rats. A significantly increased (p<0.001) TBARS level was observed in L group animals as compared to S group animals. This increase was significantly (p<0.05) attenuated in rutin pre-treated R+L group animals. No significant difference was observed between sham and R+S group animals (Fig. 3).

Effect of rutin on reduced glutathione (GSH):
The level of GSH was depleted significantly (p<0.01) in L group animals as compared to S group animals. Pre-treatment with rutin has increased its level significantly (p<0.05) in R+L group animals as compared to L group animals. There was no significant difference between R+S and S groups animals (Fig. 4).

Fig. 3: Effect of rutin pre-treatment on TBARS content in the hippocampus of ICV-STZ infused rats. Values are expressed as mean ± S.E.M. TBARS content was significantly increased in the L group as compared to S group (***p<0.001 L vs. S group). Rutin pre-treatment significantly decreased TBARS content in the R+L group animals as compared with the L group animals (#p<0.05 L vs. R+L group).
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**Fig. 4**: Effect of SAC pre-treatment on GSH level in the hippocampus of ICV-STZ infused rats. Values are expressed as mean±S.E.M. GSH level was significantly decreased in the L group as compared to S group (** p<0.01 L vs. S group). Rutin pre-treatment significantly increased GSH level in the R+L group animals as compared with the L group animals (# p<0.05 L vs. R+L group).

**Effect of rutin on nitrite level:**
The level of nitrite was significantly (p<0.001) significantly in L group animals as compared to S group animals. However animals pre-treated with rutin has shown significantly decreased (p<0.05) level of nitrite when compared with L group animals (Fig. 5).

**Effect of rutin on the activity of Poly (ADP-ribosyl) polymerase (PARP):**
The activity of PARP was normalized with respect to S group. Increased activity of PARP was found in L group animals. Rutin pre-treatment has limited the activity of PARP significantly (p<0.05) in R+L group animals as compared to L group animals (Fig. 6).
Fig. 5: Effect of rutin pre-treatment on nitrite level in the hippocampus of ICV-STZ infused rats. The level of nitrite was significantly increased in L group as compared to S group (**p<0.001 L vs. S group). Rutin pre-treatment significantly decreased nitrite level in R+L group as compared with L group animals (#p<0.05 L vs. rutin+L group). Values are expressed as mean±S.E.M.

Fig. 6: Effect of rutin pre-treatment on PARP activity in the hippocampus of ICV-STZ infused rats. The PARP activity was increased in L group animals as compared to S group animals, which was attenuated significantly in rutin pre-treated group (#p<0.05 L vs. R+L group). Values are expressed as mean±S.E.M.
Effect of rutin on antioxidant enzymes activity:

The activities of antioxidant enzymes, GPx, GR and catalase were significantly decreased in L group animals as compared to S group animals. Rutin pre-treatment has increased their activities significantly in R+L group animals as compared to L group animals. No significant change was observed in R+S group animals as compared to S group animals (Table 1).

<table>
<thead>
<tr>
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<th>S</th>
<th>L</th>
<th>R+L</th>
<th>R+S</th>
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<td>GPx (nmol NADPH oxidized min⁻¹ mg⁻¹ protein)</td>
<td>420.16±41.20</td>
<td>223.47±22.63**</td>
<td>354.21±37.37# (36.91%)</td>
<td>393.33±26.07 (-6.38%)</td>
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<tr>
<td>GR (nmol NADPH oxidized min⁻¹ mg⁻¹ protein)</td>
<td>807.24±69.54</td>
<td>410.49±30.2*** (-49.14%)</td>
<td>700.38±44.17# (41.39%)</td>
<td>755.23±36.83 (-6.44%)</td>
</tr>
<tr>
<td>Catalase (nmol of H₂O₂ consumed/mg protein)</td>
<td>83.92±7.92</td>
<td>22.71±3.44*** (-72.93%)</td>
<td>50.64±2.77## (55.15%)</td>
<td>75.97±5.37 (-9.47%)</td>
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Table 1: Values are expressed as mean ± S.E.M. STZ infusion leads to significant alterations on the activities of antioxidant enzymes (GPx, GR and Catalase) in hippocampus in L group as compared to S group animals. Pre-treatment of rutin has significantly attenuated the activity of these enzymes in R+L group animals as compared to L group animals. Values in parentheses show the percentage increase or decrease with respect to their control (*p<0.05, **p<0.01, ***p<0.001 L Vs S; #p<0.05, ##p<0.01, ###p<0.001 L Vs rutin+L)

Effect of rutin on COX-2, GFAP and NF-κB expression:

Negligible COX-2-immunopositive neurons are seen in S group animals. Prominent COX-2 expression was found in L group animals as compared to S group animals in CA1 region of hippocampus, which was decreased in R+L group animals as compared to L group animals (Fig.8). Lesion group animals revealed higher expression of GFAP immunoreactive astrocyte in the CA1 region of hippocampus as compared with S group animals. This hyper expression of astrocyte was attenuated by the pre-treatment with rutin in R+L group animals (Fig.9). The nuclear phospho-p65 NF-κB immunopositive neurons were stained in L group while in S group no nuclear phospho-p65 NF-κB staining was detected in CA1 region of hippocampus. Remarkably less nuclear phospho-p65 NF-κB staining was observed in pre-treated R+L group animals as compared to L group animals (Fig.10). Pre-treatment with rutin did not show any marked effects on COX-2, phospho-p65 NF-κB and GFAP expression in the R+S group animals as compared to sham group animals (data not shown).
Fig. 7: Histopathological changes in the CA1 region of hippocampus. Sections were stained with hematoxylin and eosin. White arrows indicate the normal pyramidal neuron in S group (B) and black arrows indicate the degenerated pyramidal neuron in L group (D) while L group pretreated with rutin shows normal pyramidal neuron staining (F), with less degenerated neurons.
Fig. 8: Effect of rutin pre-treatment on COX-2 expression in CA1 region of hippocampus in ICV-STZ infused rats. Almost negligible staining of COX-2 was observed in S group (A, B) while over-expression of COX-2 was observed in L group(C, D). Lesion group pre-treated with rutin showed moderate staining of COX-2 expression (E, F) Magnification 10X (A,C,E) 40X (B, D, F)
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Effect of rutin on iNOS and IL-8 expression (Immuno-fluorescence detection):

Higher expression of iNOS (Fig. 11) and IL-8 (Fig. 12) was seen in L group as compared to S group animals in CA1 region of hippocampus. Rutin pretreatment attenuated higher expression of iNOS and IL-8 in R+L group animals as compared to L group animals.
Effect of rutin on the morphological changes of pyramidal neuron in the CA1 region of hippocampus:

S group animals show typical histopathological architecture of pyramidal neurons in CA1 region of the hippocampus. While abnormal as well as degenerated neurons were seen in L
group animals as compared to S group animals. Although pre-treatment with rutin reduced STZ induced histological abnormalities in R+L group animals (Fig. 7).

![Image of group animals comparison](image1)

Fig. 11: Effect of rutin pre-treatment on iNOS expression in CA1 region of hippocampus in ICV-STZ infused rats. Over expression of iNOS was found in L group as compared to S group animals while rutin pre-treatment lessen the effect of over expression of iNOS in R+L group animals. Magnifications 40X

![Image of iNOS expression](image2)

Fig. 12: Effect of rutin pre-treatment on interleukin-8 (IL-8) expression in CA1 region of hippocampus in ICV-STZ infused rats. Over expression of IL-8 were observed in lesion group (B) as compared to S group animals (A) while rutin pre-treatment lessen the effect of over expression of IL-8 in R+L group animals (C). Magnifications 40X.

**Discussion:**

The present study examined the effect of rutin on memory impairment, oxidative stress, and neuroinflammation in ICV-STZ injected rats. The bilateral ICV infusion of streptozotocin caused learning and memory deficits and oxidative stress in line with the earlier findings (Ishrat et al., 2009a, 2009b; Khan et al., 2006; Javed et al., 2011). Our results exhibited that pretreatment with rutin retarded brain injury subsequent to ICVSTZ injection in rats. The
antioxidant and neuroprotective potential of rutin may contribute to this effect by diminishing oxidative stress. These findings are in harmony with the earlier study carried out by us and others (Bishnoi et al., 2007).

Cholinergic function is important for the learning and memory and its alteration play a key role in the development of cognitive impairment. Morris water maze test was employed in the present study to test the spatial learning and memory of animals. In this behavioural test the animal’s escape from the water reinforces its desire to find the submerged platform quickly, and on subsequent trails the animal would be able to locate the platform more rapidly. So such improvement in performance occurs because the animals have learned where the hidden platform is located in the water pool relative to the conspicuous visual cues. Our Morris water maze data presented that, ICV-STZ infused rats travelled long distance (path length) and took longer time (escape latency) to reach the submerged platform, indicating poorer learning and memory process which is consistent with the previous reports (Ishrat et al., 2009a, 2009b; Khan et al., 2006; Tota et al., 2011). Moreover, rutin administration significantly ameliorated the deficits in learning and memory in ICV-STZ rats as showed by reduced path length and escape latency to reach the hidden platform. Our findings are in harmony with others findings carried out by others where rutin supplementation is effective in retarding memory dysfunction resulting from hippocampal neuron loss such as in Alzheimer’s disease (Pu et al., 2004).

Increasing evidences suggest that the excessive production of free radicals in brain and the imbalance between oxidative species and antioxidant defences is related to aging and the pathogenesis of neurodegenerative diseases (Schulz et al., 2000; Kasapoglu and Ozben, 2001). Glutathione redox cycling is an extremely important in cellular free radical detoxification. Glutathione (GSH) homeostasis is maintained by glutathione synthesis and redox cycling. GSH is the most abundant non-protein thiol that buffers ROS in the brain tissue (Dringen et al., 2000). It removes H₂O₂ and other organic peroxides with the help of glutathione peroxidase (GPx) (Meister, 1988). During detoxification process, oxy-radicals are reduced by GPx to form glutathione disulfide (GSSG). GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by glutathione reductase (GR) with the consumption of one NADPH. Reduction in the level of GSH hampers free radical clearance in the brain leading to more oxidant pack and consequently oxidative stress. Thus, the antioxidant enzymes, GPx, and GR are responsible for the maintenance of redox cycle of GSH which in turn play an important role in the detoxifying of ROS (Ansari et al., 2008). Decreased activity of GPx and GR directly affect the level of GSH resulting overall low level
of antioxidant system, thus predicting the massive load of ROS in AD (Ansari et al., 2008). Increased level of \( \text{H}_2\text{O}_2 \) induces the oxidation of polyunsaturated fatty acids leading to lipid peroxidation (Dringen et al., 2000). Lipid peroxides further leads to producing of more stable compound like malondialdehyde (MDA), 4-hydroxy-trans-2-nonenal (4-HNE) and acrolein which results the oxidative stress. Catalase is found at a very low amount in the brain, responsible for the detoxification of extremely toxic compound \( \text{H}_2\text{O}_2 \) into \( \text{O}_2 \) and \( \text{H}_2\text{O} \). So the decreased activity of catalase is very critical for the brain. In the present study it was observed that ICV-STZ administration causes lipid peroxidation, as evidenced by increased TBARS content, and decreased level of GSH and activities of antioxidant enzymes in ICV-STZ infused animals, leading to neurodegeneration. Rutin supplementation significantly counteracted all the changes in the markers of oxidative stress. This occurs often due to rutin’s free-radical-scavenging and neuroprotective properties (Kamalakkannan and Prince, 2006; Bishnoi et al., 2007; Khan et al., 2009). Rutin also modulate the permeability of the walls of the blood vessels including capillaries and protect the neuronal loss which is supported by the histopathological study of lesion group animals as compared to R+L group animals. Oxidative neuronal insult in ICV-STZ infused rats is consistent with our previous findings (Ishrat et al., 2006; Khan et al., 2006).

Under normal physiological conditions, poly (ADP-ribose) polymerase-1 (PARP-1) is the routine repair of DNA damage by adding poly (ADP-ribose) polymers in response to a variety of cellular stresses. Recently, it was reported that PARP-1 participates in diverse range of physiological and pathological functions from cell survival to cell death and has been implicated in immune responses, inflammation, angiogenesis, gene transcription, learning, memory and aging (Chaitanya et al., 2010). Oxidative-nitrosative stress induced massive DNA damage leads to hyper activation of PARP-1 that initiates high consumption of \( \text{NAD}^+ \) and consequently leads to depletion of cellular energy. Activated PARP-1 is a mediator of neuron death during excitotoxicity, ischemia, and oxidative stress (Alano et al., 2010). We observed higher activity of PARP-1 in lesion group while rutin supplementation restores PARP activity significantly in rutin pretreated group animals (R+L). Oxidative stress associated PARP-1 activation regulate the inflammatory responses. The ability of PARP-1 to regulate inflammation is suggested to be due to its co-activation of nuclear transcription factor NF-\( \kappa \)B. NF-\( \kappa \)B responds directly to reactive oxygen species and \( \text{H}_2\text{O}_2 \) (Schreck et al., 1991; Schmidt et al., 1995). This transcription factor play important role in the pathogenesis of oxidative stress-associated neurodegenerative disorders (Frank et al., 1998). NF- \( \kappa \)B is usually kept inactive in the cytoplasm by its inhibitor protein (I-\( \kappa \)B), but
IkB is degraded on receipt of cellular stress, leading to phosphorylation and nuclear translocation, subsequently binds to DNA and induce the transcription of targeted genes such as TNF-α, IL-1β, iNOS, COX-2, cytokines like IL-8, adhesion molecules that may execute the inflammatory reactions (Shen et al., 2010). In the present study we observed higher expression of phospho-p65 nuclear NF-κB in lesion group animals in CA1 region of hippocampus. Rutin pretreated group (R+L) showed remarkably less phospho-p65 nuclear NF-κB expression, suggesting the involvement of such NF-κB modulation in the anti-inflammatory effect of rutin (Kamalakkannan and Prince, 2006).

In brain COX-2 expressed in distinct population of neurons (Breder et al., 1995) which makes the understanding common of enzymes involved in physiological functions of the central nervous system such as memory, sensory integration, and autonomic regulation and may suggest its role (Kaufmann et al., 1997). The most important mechanisms associated with the toxic effects of the enhanced COX-2 activity during neuroinflammation are the production of prostaglandin E2 (Candelario-Jalil et al., 2007) and formation of free radicals leading to oxidative stress (Candelario-Jalil et al., 2006). Higher expression of COX-2 mRNA and protein was found in the AD brains (Yasojima et al., 1999). Our study presented higher expression of COX-2 in the CA1 region of hippocampus in lesion group which was lessen by the rutin administration. This evidence can make rutin as a promising therapeutic candidate for the inflammation induced neurodegenerative disorder.

It was shown that reactive oxygen and reactive nitrogen intermediates may in turn stimulate to synthesize IL-8 (Remick and Villarete, 1996). Several other toxic stimulants may also have the potential to stimulate the astrocyte or microglia to secrete the cytokines such as IL-8. It is reported that interleukins, IL-1, IL-6 and IL-8 are synthesized by activated microglia and macrophages in response to pathogens or the occurrence of trauma (Dunn, 1991). These interleukins normally recruit more microglia and macrophages to the affected site, thereby coordinating the immune response, regulating tissue regrowth, and promoting wound healing. Since interleukins are synthesized and secreted as needed, and are not stored, they typically have short life. However, they can damage central nervous system when they are secreted over a prolonged period, in part because they recruit and activate macrophages that produce high concentration of reactive oxygen species (Dunn, 1991). IL-8 has also been shown to function as a trophic factor in the maintenance of normal neuronal populations and promotion of neuron survival (Limatola et al., 2000). Our results shows, higher expression of IL-8 in lesion group as compared to sham group animals that is close to up regulated GFAP positive...
astrocytic expression in lesion group. However, pre-treatment with rutin decreased the expression of IL-8 and GFAP in R+L group animals.

It is well documented that inducible nitric oxide synthase (iNOS) produces nitric oxide (NO) and NO-derived reactive nitrogen species such as peroxynitrite. In healthy neuronal tissue iNOS is not commonly present, but it can be expressed by astrocyte, neurons and endothelial cells after brain offence, where it can initiate the production of high amounts of NO (Vallance and Leiper, 2002). Overproduction of NO may lead to neuronal damage and death. The reaction between NO and super oxide anion generate the cytotoxic compound, peroxynitrite that leads to neuronal toxicity (Vallance and Leiper, 2002). Under normal physiological conditions, antioxidant enzymes are responsible to eliminate the highly reactive molecules (Thannickal and Fanburg, 2000). However, under unphysiological conditions, the excessive accumulation of reactive species induces several cellular dysfunctions (Thannickal and Fanburg, 2000). The present data revealed that ICV-STZ injection induced a marked iNOS expression that was close to higher level of nitrite, which served as indicator of NO production (Green et al., 1982) in lesion group. Interestingly rutin supplementation decreases iNOS expression and nitrite level in rutin pretreated group.

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