CHAPTER 3

THERAPEUTIC ROLE OF GINSENG AND QUERCETIN AGAINST EXPERIMENTAL MODEL OF MILD TRAUMATIC BRAIN INJURY INDUCED COGNITIVE LOSS

CHAPTER 3.1: NITRIC OXIDE MODULATORY MECHANISM OF GINSENG AGAINST MILD TRAUMATIC BRAIN INJURY INDUCED COGNITIVE IMPAIRMENT

3.1.1 INTRODUCTION

Traumatic brain injury due to accidents is considered to be a challenging public health problem (Prins and Hovda, 2003). It is a leading cause of death and disability which accounts for approximately 2.5 to 6.5 million cases in the United States (Maas et al., 2008). It is a common neurological impairment known to trigger a variety of symptoms ranging from simple headache to permanent cognitive dysfunction. Brain injury can be classified as primary, which occurs immediately after trauma, and secondary, which includes a cascade of injuries that develop over a period of time after the initial traumatic episode (Raghupathi et al., 2002). Survivors of head trauma suffer from a wide variety of pathologies such as neurological deficits, behavioral and emotional impairments, weakened motor function, aggression, seizures and cognitive problems, all of which depend on the severity of the injury (Masel and DeWitt, 2010). Head injury is known to cause significant damage to the hippocampal cells, which plays a crucial role in the processing of spatial learning and memory (Tong et al., 2002). A clinical report also shows that head trauma survivor’s faces difficulty in spatial learning (Skelton et al., 2000). Oxidative damage and apoptosis are two pathogenic mechanisms that play a significant role in the progression of secondary brain injury (Bayir et al., 2003). Damage caused by oxidative cellular injury and activated inflammatory response have been well known (Halliwell, 2006). The existence of a vicious cycle involving free radicals and inflammatory cytokines leading to neuronal death in post-traumatic stress disorder has also been well
demonstrated (Pall and Satterlee, 2001). However, the exact cellular or molecular cascade causing oxidative damage and apoptotic neurodegeneration in brain trauma is not very clear.

Nitric oxide (NO) is an important neurotransmitter involved in many neurological disorders and plays a crucial role in cell signalling (Garthwaite, 1991). NO is known to participate in several neurobiological functions of brain including learning and memory (Boehning and Snyder, 2003). These deficits are known to get aggregated by NO donors such as L-arginine. On the other hand, L-NAME (NG-nitro-L-arginine methyl ester) is known to attenuate NOS activity and reduces its deleterious effects (Yamada et al., 1996). Recent report suggests an abundant association of nitric oxide synthase with neurobiology of memory functions and its relation with pathological mechanisms of the disease (Javadi-Paydar et al., 2013).

Since, there are no specified pharmacological agents that could block the progression of the secondary injury; the current management of brain trauma cases are merely symptomatic. Since, oxidative stress and neuroinflammation are major contributors in the pathogenesis of brain injury therefore; the search for a new therapeutic antioxidant and anti-inflammatory agent is essentially required to be investigated. American Ginseng (AG) (Panax quinquefolium, family Araliaceae), has been popularly used as a tonic herb from around 2000 years in eastern countries. Presently, AG is one of the most famous and consumed herbal medicines all around the world. The major active constituents of the ginseng are triterpenoid saponins (ginsenosides), which are four-ringed steroidal structure, responsible for its therapeutic effect on the central nervous system (Nah et al., 2007). Recently, ginseng root extract has improved learning and memory performance in scopolamine induced cognitive dysfunction (Al-Hazmi et al., 2013). Earlier report has also shown that ginsenoside Rb1 can protect hippocampal neurons against ischemic conditions (Lim et al., 1997). Ginsenoside Rg1 has been known to exert neuroprotective activities via its anti-oxidative effects in cultured neurons (Liu et al., 2011). Wu and its group have reported that ginsenosides-Re has anti-inflammatory effect and inhibits microglial cells activation and generation.
of pro-inflammatory cytokines (Wu et al., 2007). All these data clearly explains the neuroprotective potential of AG; however, its exact cellular or molecular mechanism is still not clearly understood.

Therefore, the present study has been designed to investigate the neuroprotective mechanism of American ginseng and the possible involvement of nitric oxide mechanism against traumatic brain injury-induced cognitive impairment.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Animals

Adult male Wistar rats (200–250 g) were procured from Animal House of Panacea Biotec Ltd, Lalru (Panjab). Animals were housed under standard laboratory conditions (25±2°C, 60–70% humidity) and maintained on a 12 hour natural day–night cycle, with free access to food and water. Animals were acclimatized to laboratory conditions before experimental tests. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Panjab University (IAEC/170-175/UIPS/16) and conducted according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India on the use and care of experimental animals.

3.1.2.2 Mild traumatic brain injury (mTBI)

A special weight-drop device developed by Marmarou and its group (Marmarou et al., 1994) and modified as described by Pandey and its group (Pandey et al., 2009) was used to deliver a standard diffuse traumatic impact (Picture 3.1.1). Under ketamine and xylazine anesthesia (75 and 5 mg/kg, i.p.), a midline incision (1 cm) was made on the scalp of rats and skin was retracted to expose the skull. A 10 mm diameter, 3 mm thick metallic disk designed to protect against skull fracture was placed between bregma and lambda suture lines of the skull. A cylindrical metallic weight of 450 g was dropped from a height of 2 m freely through a metal tube onto the disc. Foam bed (10 cm) was placed underneath the animal to absorb the impact due to weight drop. After impact, the metal disc was removed and skin was sutured with absorbable sutures (Ethicon 4-0, Absorbable surgical sutures USP...
To prevent the surgical wound, povidone-iodine (10% w/v, Betadine) was applied to the wound closure site. Sham operated rats were treated in the similar way, including mid-line incision, except brain injury. To prevent post surgical infection, the animals received Sulprim injection (each ml containing 200 and 40 mg of sulphadiazine and trimethoprim respectively), intramuscularly (0.2 ml/300 g) once a day for 3 days of post-surgery. Brain injured and sham-operated animals were housed singly in cages to prevent any disturbance. All the animals from day 0-14 were given continuous care with daily observation and handling.

**Picture 3.1.1** Weight drop device for mild traumatic brain injury

3.1.2.3 Drugs and treatment schedule

American ginseng, L-NAME and L-Arginine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). ELISA kits for TNF-α and caspase-3 was purchased from R&D Systems (USA). The animals were
randomly divided into ten experimental groups (n=5-6). First and second group was named as naïve and sham group respectively. Third group was named as mTBI (mild traumatic brain injury) group. American ginseng (AG) (50, 100 and 200 mg/kg) were treated as group 4-6 respectively. Treatment of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) was categorized as group 7 and 8 respectively. Pretreatment of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) with AG (100 mg/kg) served as group 9-10. American ginseng (AG) extract was prepared in peanut oil where as L-NAME and L-arginine was dissolved in normal saline and administered orally (30 min before AG treatment) on the basis of body weight (0.5 ml/100 g). Pictogram of the entire protocol is represented in Fig. 3.1.1.

3.1.2.4 Behavioral Assessment

3.1.2.4.1 Morris water-maze test

Animals were tested individually in a spatial version of Morris water maze test (Morris et al., 1982) from day 24–28. The apparatus consists of a
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circular water tank (180 cm in diameter and 60 cm high). A platform (12.5 cm in diameter and 38 cm high) invisible to animals, was set 2 cm below the water level inside the tank with water maintained at 28.5 ± 2°C at a height of 40 cm. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the animals for spatial orientation. The position of the cues remained unchanged throughout the study. The animals received four consecutive daily training trials in the following 5 days, with each trial having a ceiling time of 120 s and a trial interval of approximately 30 min. For each trial, each animal was put into the water at one of four starting positions, the sequence of which being selected randomly. During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall. The animal had to swim until it climbed onto the platform submerged underneath the water. After climbing onto the platform, the animal remained there for 20 s before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the animal failed to reach the escape platform within the maximally allowed time of 120 s, it was guided with the help of a rod and allowed to remain on the platform for 20 s. The time to reach the platform (escape latency in seconds), time spent and frequency of appearance in target quadrant and total distance travelled to reach the hidden platform (path length in cm) was measured by using computer tracking system with Ethovision software, Noldus Information Technology, The Netherlands.

Memory retrieval test

A probe trial was performed at the end of 28th day wherein the extent of memory retention was assessed. In the probe trial, the rats were placed into the pool for a total duration of 120 s as in the training trial, except that the hidden platform was removed from the pool. Various parameters such as time spent in target quadrant (TSTQ), frequency of appearance in target quadrant, and % of total path length traversed in target quadrant were measured using computer tracking system with Ethovision software.
3.1.2.5 Serum corticosterone estimations

3.1.2.5.1 Preparation of serum
As per section (1.1.2.5.1)

3.1.2.5.2 Corticosterone assessment
As per section (1.1.2.5.2)

3.1.2.6 Biochemical assessments

Immediately after the last behavioral test, animals were sacrificed by cervical dislocation and brain samples were rapidly removed and placed on dry ice for dissection of brain hippocampus region. A 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × g for 15 min. Aliquots of supernatants were separated and used for biochemical and cellular estimations.

3.1.2.6.1 Assessment of oxidative stress
As per section (1.1.2.6.1)

3.1.2.6.1.1 Estimation of lipid peroxidation
As per section (1.1.2.6.1.1)

3.1.2.6.1.2 Estimation of reduced glutathione
As per section (1.1.2.6.1.2)

3.1.2.6.1.3 Estimation of superoxide dismutase
As per section (1.1.2.6.1.3)

3.1.2.6.1.4 Estimation of catalase
As per section (1.1.2.6.1.4)

3.1.2.6.1.5 Protein estimation
As per section (1.1.2.6.1.5)
3.1.2.6.2 Assessment of nitrosative stress

As per section (1.1.2.6.2)

3.1.2.7 Molecular estimations

3.1.2.7.1 Estimation of tumor necrosis factor-alpha (TNF-\(\alpha\))

As per section (2.1.2.7.1)

3.1.2.7.2 Estimation of apoptotic factor (caspase-3)

Caspase-3, also known as CPP-32 is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase specific peptide that is conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysates/homogenates is directly proportional to the color reaction. The enzymatic reaction for caspase activity was carried out using R&D systems caspase-3 colorimetric kit.

3.1.2.8 Statistical analysis

As per section (2.1.2.8)

3.1.3 RESULTS

3.1.3.1 Effects of American ginseng (AG) on escape latency time (ELT) in Morris water maze test and its interaction with nitric oxide modulators

mTBI resulted in a significant delay in ELT to reach the hidden platform from day 24-27 as compared to the sham group, depicting cognitive dysfunction (p<0.05). Sham treatment did not show any significant effect on ELT as compared to naïve group. AG (50 mg/kg) treatment for 14 days did not show any significant effect on ELT as compared to mTBI control. Further, AG (100, 200 mg/kg) treatment for 14 days significantly shortened ELT as compared to mTBI control (p<0.05). In addition, pretreatment of L-NAME (10
mg/kg) with subeffective dose of AG (100 mg/kg) for 14 days potentiated its protective effects (decreased escape latency time) which was also significant as compared to their effects alone in mTBI treated rats (P<0.05). However, L-arginine (100 mg/kg) pretreatment with AG (100 mg/kg) significantly reversed the protective effect of AG (100 mg/kg). Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on ELT as compared to sham (data not shown) (Fig. 3.1.2).

![Graph showing effects on escape latency time](image)

**Fig. 3.1.2 Effects of American ginseng (AG) on escape latency time in Morris water maze test and its interaction with nitric oxide modulators.** Values are expressed as mean ± SEM. For statistical significance, ¹P <0.05 as compared to sham group; ²P <0.05 as compared to mTBI; ³P <0.05 as compared to mTBI+AG(50); ⁴P <0.05 as compared to mTBI+AG(100); ⁵P <0.05 as compared to MTBI+L-NAME(10) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; AG(50, 100, 200), American ginseng (50, 100, 200 mg/kg); L-ARG, L-Arginine
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3.1.3.2 Effects of American ginseng (AG) on frequency and time spent in target quadrant (TSTQ) and its interaction with nitric oxide modulators

mTBI treatment significantly reduced frequency and time spent in target quadrant (TSTQ) during probe trial (day 28) as compared to sham (p<0.05). Sham treatment did not show any significant effect on frequency and TSTQ as compared to naive. AG (100, 200 mg/kg) treatment for 14 days significantly improved frequency (Fig. 3.1.3.1) and TSTQ (Fig. 3.1.3.2) as compared to control. Further, L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatment did not show any significant effect on frequency and TSTQ as compared to mTBI control. However, L-NAME (10 mg/kg) pretreatment with subeffective dose of AG (100 mg/kg) for 14 days significantly potentiated their protective effect (increased frequency and TSTQ) as compared to their effects alone. However, L-arginine (100 mg/kg) pretreatment with AG (100 mg/kg) significantly reversed the protective effect of AG (100 mg/kg) in mTBI treated rats (p<0.05). Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on frequency and TSTQ as compared to sham (data not shown).

Fig. 3.1.3.1 Effects of American ginseng (AG) on frequency of appearance in target quadrant and its interaction with nitric oxide modulators. Values are expressed as mean ± SEM. For statistical significance, \(^{a}P<0.05\) as compared to sham group; \(^{b}P<0.05\) as compared to mTBI; \(^{c}P<0.05\) as compared to mTBI+AG(50); \(^{d}P<0.05\) as compared to mTBI+AG(100); \(^{e}P<0.05\) as compared to MTBI+L-NAME(10) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; AG(50, 100, 200), American ginseng (50, 100, 200 mg/kg); L-ARG, L-Arginine.
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3.1.3.2 Effect of American ginseng (AG) on time spent in target quadrant (TSTQ) and its interaction with nitric oxide modulators.

Fig. 3.1.3.2 Effects of American ginseng (AG) on time spent in target quadrant (TSTQ) and its interaction with nitric oxide modulators. Values are expressed as mean ± SEM. For statistical significance, \( ^{a}P < 0.05 \) as compared to sham group; \( ^{b}P < 0.05 \) as compared to mTBI; \( ^{c}P < 0.05 \) as compared to mTBI+AG(50); \( ^{d}P < 0.05 \) as compared to mTBI+AG(100); \( ^{e}P < 0.05 \) as compared to MTBI+L-NAME(10) (One-way ANOVA followed by Tukey’s test).

3.1.3.3 Effect of American ginseng (AG) on path length and its interaction with nitric oxide modulators

The total distance travelled to reach the hidden platform (path length) significantly increased in mTBI treated rats as compared to sham animals (p<0.05). Sham treatment did not show any significant effect as compared to naive. AG (100, 200 mg/kg) treatment for 14 days significantly shortened path length as compared to mTBI control (p<0.05). Further, L-NAME (10 mg/kg) pretreatment with AG (100 mg/kg) potentiated its protective effects which was significant as compared to their effects alone (p<0.05). L-arginine (100 mg/kg) pretreatment significantly reversed the protective effect of AG (100 mg/kg). Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on path length as compared to sham (data not shown) (Table 3.1.1). Further, the computer tracking of the path travelled to reach the hidden platform is shown in Fig 3.1.4.
Table 3.1.1 Effects of American ginseng (AG) on the total distance travelled to reach the hidden platform (path length) and its interaction with nitric oxide modulators

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Path length (cm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 4</td>
</tr>
<tr>
<td>Naive</td>
<td>2154.2±98.5</td>
<td>1356.4±121.2</td>
<td>1012.3±123.4</td>
<td>823.1±83.4</td>
</tr>
<tr>
<td>Sham</td>
<td>2165.4±133.3</td>
<td>1365.3±142.3</td>
<td>1034.5±137.6</td>
<td>833.4±94.3</td>
</tr>
<tr>
<td>mTBI</td>
<td>2212.3±131.2</td>
<td>2124.2±122.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2034.2±125.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1945.1±143.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>mTBI + AG(50)</td>
<td>2143.1±151.7</td>
<td>2054.6±143.1</td>
<td>1952.4±118.4</td>
<td>1865.4±112.3</td>
</tr>
<tr>
<td>mTBI + AG(100)</td>
<td>2202.8±123.4</td>
<td>1784.6±153.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1678.3±131.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1324.5±143.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>mTBI + AG(200)</td>
<td>2134.4±121.3</td>
<td>1462.4±124.6&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1243.5±117.7&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1045.6±94.5&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>mTBI + L-NAME(10)</td>
<td>2134.4±122.4</td>
<td>2082.3±142.5</td>
<td>2012.4±105.6</td>
<td>1912.4±113.4</td>
</tr>
<tr>
<td>mTBI + L-ARG(100)</td>
<td>2203.5±107.4</td>
<td>2123.4±111.5</td>
<td>2054.6±93.6</td>
<td>1955.3±131.2</td>
</tr>
<tr>
<td>mTBI + L-NAME(10) + AG (100)</td>
<td>2209.2±112.4</td>
<td>1498.5±131.9&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>1284.6±131.4&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>1099.4±128.8&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>mTBI + L-ARG(10) + AG (100)</td>
<td>21145±115.3</td>
<td>2004.5±133.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1931.5±152.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1821.1±123.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, <sup>a</sup>P <0.05 as compared to sham group; <sup>b</sup>P <0.05 as compared to mTBI; <sup>c</sup>P <0.05 as compared to mTBI+AG(50); <sup>d</sup>P <0.05 as compared to mTBI+AG(100); <sup>e</sup>P <0.05 as compared to mTBI+L-NAME(10) (One-way ANOVA followed by Tukey's test). mTBI, mild traumatic brain injury; AG(50, 100, 200), American ginseng (50, 100, 200 mg/kg); L-ARG, L-Arginine.
3.1.3.4 Effect of American ginseng (AG) on serum corticosterone (CORT) and its interaction with nitric oxide modulators

Mild traumatic brain injury (mTBI) significantly increased serum CORT level as compared to sham group (p<0.05). Sham treatment did not show any significant effect on serum CORT level as compared to naïve group. Treatment with American ginseng (AG) (50 mg/kg) for 14 days did not show any significant effect on serum CORT level as compared to mTBI control. American ginseng (AG) (100, 200 mg/kg) treatment for 14 days significantly attenuated serum CORT level as compared to mTBI control. Further, L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatment did not show any significant effect on serum CORT level as compared to control. However, L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatment did not show any significant effect on serum CORT level as compared to control.
mg/kg) pretreatment with subeffective dose of American ginseng (AG) (100 mg/kg) for 14 days potentiated its protective effect (reduced serum CORT level) which was significant as compared to their effects alone (p<0.05). However, L-arginine (100 mg/kg) pretreatment significantly reversed the protective effect of American ginseng (AG) (100 mg/kg) in mTBI treated rats. Further, per se effect of American ginseng (AG) (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on serum corticosterone (CORT) level as compared to sham (data not shown) (Fig. 3.1.5).

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Fig. 3.1.5 Effect of American ginseng (AG) on serum corticosterone (CORT) levels and its interaction with nitric oxide modulators. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+AG(50); dP <0.05 as compared to mTBI+AG(100); eP <0.05 as compared to mTBI+L-NAME(10) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; AG(50, 100, 200), American ginseng (50, 100, 200 mg/kg); L-ARG, L-Arginine.
3.1.3.5 Effect of American ginseng (AG) on oxidative-nitrosative stress in brain hippocampus and its interaction with nitric oxide modulators

mTBI treatment significantly increased oxidative stress markers in brain hippocampus as evidenced by increase in lipid peroxidation (LPO), and depletion of reduced glutathione (GSH), catalase and superoxide dismutase (SOD) level as compared to sham group (p<0.05). Apart from oxidative damage, mTBI caused a significant increase in nitrosative stress marker as seen by increased nitrite concentration as compared to sham (p<0.05). Sham treatment did not show any significant effect on oxidative-nitrosative stress as compared to naïve group. Treatment with American ginseng (AG) (50 mg/kg) for 14 days did not show any significant effect on oxidative-nitrosative stress markers as compared to mTBI control. However, treatment with American ginseng (AG) (100, 200 mg/kg) for 14 days significantly attenuated oxidative-nitrosative stress markers (reduced lipid peroxidation and nitrite levels, and restored reduced glutathione, catalase and superoxide dismutase level) as compared to mTBI control (p<0.05). However, L-NAME (10 mg/kg) pretreatment with subeffective dose of American ginseng (AG) (100 mg/kg) for 14 days potentiated their protective effect (reduced lipid peroxidation and nitrite levels, and restored reduced glutathione, catalase and superoxide dismutase level) which was significant as compared to their effects alone. However, pretreatment of L-arginine (100 mg/kg) with American ginseng (AG) (100 mg/kg) significantly reversed the protective effect of American ginseng (AG) (100 mg/kg) in mTBI treated rats. Further, per se effect of American ginseng (AG) (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on lipid peroxidation, nitrite, reduced glutathione, catalase and superoxide dismutase level as compared to sham group (data not shown) (Table 3.1.2).
Table 3.1.2 Effect of American ginseng on oxidative-nitrosative stress and its interaction with nitric oxide modulators

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LPO (mole of MDA/mg pr) (% of sham)</th>
<th>GSH (umole of GSH/mg pr) (% of sham)</th>
<th>SOD (units/mg pr) (% of sham)</th>
<th>Catalase (umole of H$_2$O$_2$/min/mg pr) (% of sham)</th>
<th>Nitrite (µg/ml) (% of sham)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>0.114±0.05 (96.6)</td>
<td>0.063±0.004 (99.6)</td>
<td>1.32±0.24 (105.6)</td>
<td>0.64±0.06 (103.2)</td>
<td>245.6±10.2 (96.8)</td>
</tr>
<tr>
<td>Sham</td>
<td>0.118±0.04 (100)</td>
<td>0.065±0.003 (100)</td>
<td>1.25±0.62 (100)</td>
<td>0.62±0.04 (100)</td>
<td>253.5±8.32 (100)</td>
</tr>
<tr>
<td>mTBI</td>
<td>0.456±0.07a (400)</td>
<td>0.016±0.006a (24.6)</td>
<td>0.24±0.04a (19.2)</td>
<td>0.12±0.01a (19.4)</td>
<td>580.5±12.2a (229.2)</td>
</tr>
<tr>
<td>mTBI +AG(50)</td>
<td>0.431±0.06c (365.3)</td>
<td>0.019±0.004c (29.3)</td>
<td>0.30±0.02 (24)</td>
<td>0.18±0.05 (29.1)</td>
<td>561.2±12.2 (221.7)</td>
</tr>
<tr>
<td>mTBI +AG(100)</td>
<td>0.302±0.08h,c (255.9)</td>
<td>0.033±0.007h,c (50.8)</td>
<td>0.78±0.08h,c (62.4)</td>
<td>0.35±0.06h,c (56.5)</td>
<td>454.5±11.5h,c (179.4)</td>
</tr>
<tr>
<td>mTBI +AG(200)</td>
<td>0.173±0.05d,de (146.6)</td>
<td>0.055±0.004cd,de (84.6)</td>
<td>1.16±0.06cd,de (92.8)</td>
<td>0.58±0.04cd,de (93.5)</td>
<td>313.5±10.5cd,de (123.7)</td>
</tr>
<tr>
<td>mTBI+L-NAME(10)</td>
<td>0.447±0.08 (378.8)</td>
<td>0.018±0.002 (27.7)</td>
<td>0.28±0.10 (22.6)</td>
<td>0.14±0.02 (22.6)</td>
<td>573.5±12.5 (226.5)</td>
</tr>
<tr>
<td>mTBI +L-ARG(100)</td>
<td>0.457±0.04 (387.3)</td>
<td>0.017±0.006 (26.2)</td>
<td>0.23±0.03 (18.4)</td>
<td>0.11±0.02 (17.7)</td>
<td>584.6±12.5 (230.8)</td>
</tr>
<tr>
<td>mTBI+L-NAME(10) +AG(100)</td>
<td>0.180±0.07d,e (152.5)</td>
<td>0.052±0.007d,e (80)</td>
<td>1.09±0.11d,e (87.2)</td>
<td>0.52±0.07d,e (83.9)</td>
<td>315.6±12.5d,e (124.5)</td>
</tr>
<tr>
<td>mTBI+L-ARG(100) +AG(100)</td>
<td>0.424±0.07d (359.3)</td>
<td>0.020±0.004d (30.8)</td>
<td>0.35±0.02d (28.2)</td>
<td>0.19±0.04d (30.6)</td>
<td>546.7±11.4d (215.8)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, *P <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+AG(50); dP <0.05 as compared to mTBI+AG(100); eP <0.05 as compared to MTBI+L-NAME(10) (One-way ANOVA followed by Tukey's test). mTBI, mild traumatic brain injury; AG(50, 100, 200), American ginseng (50, 100, 200 mg/kg); L-ARG, L-Arginine.
3.1.3.6 Effect of American ginseng (AG) on acetylcholinesterase (AChE) activity and its interaction with nitric oxide modulators

mTBI treatment significantly increased acetylcholinesterase enzyme activity in brain hippocampus as compared to sham group (p<0.01). Sham treatment did not show any significant effect on AChE activity compared to naive group. AG (50 mg/kg) treatment did not show any significant effect as compared to mTBI control. However, treatment with AG (100, 200 mg/kg) significantly attenuated AChE activity as compared to mTBI control. However, L-NAME (10 mg/kg) pretreatment with AG (100 mg/kg) for 14 days potentiated its protective effect (reduced AChE activity) which was significant as compared to their effects alone (p<0.01). However, pretreatment of L-arginine (100 mg/kg) with AG (100 mg/kg) significantly reversed the protective effect of AG (100 mg/kg). Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect on AChE activity as compared to sham (data not shown) (Fig. 3.1.6).

![Graph showing effect of American ginseng (AG) on brain hippocampus acetylcholinesterase activity and its interaction with nitric oxide modulators.](image)

**Fig. 3.1.6** Effect of American ginseng (AG) on brain hippocampus acetylcholinesterase activity and its interaction with nitric oxide modulators. Values are expressed as mean ± SEM. For statistical significance, *P <0.05 as compared to sham group; ^P <0.05 as compared to mTBI; *P <0.05 as compared to mTBI+AG(50); *P <0.05 as compared to mTBI+L-NAME(10); *P <0.05 as compared to MTBI+L-NAME(10) (One-way ANOVA followed by Tukey’s test).
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3.1.3.7 Effect of American ginseng (AG) on tumor necrosis factor (TNF-α) and apoptotic factor (caspase-3) and its interaction with nitric oxide modulators

mTBI treatment significantly increased TNF-α and caspase-3 level in brain hippocampus as compared to sham group (p<0.05). Sham treatment did not show any significant effect on TNF-α and caspase-3 level as compared to naive. AG (50 mg/kg) treatment did not show any significant effect on TNF-α (Fig. 3.1.7.1) and caspase-3 (Fig. 3.1.7.2) level as compared to control. However, AG (100, 200 mg/kg) significantly reduced TNF-α and caspase-3 level compared to control. However, L-NAME (10 mg/kg) pretreatment with AG (100 mg/kg) potentiated its protective effect which was significant as compared to their effects alone. Further, pretreatment of L-arginine (100 mg/kg) with AG (100 mg/kg) significantly reversed the protective effect of AG. Further, per se effect of AG (400 mg/kg), L-NAME (10 mg/kg) and L-arginine (100 mg/kg) did not show any significant effect as compared to sham (data not shown).

Fig. 3.1.7.1 Effect of American ginseng (AG) on tumor necrosis factor (TNF-α) level and its interaction with nitric oxide modulators. Values are expressed as mean ± SEM. For statistical significance, aP<0.05 as compared to sham group; bP<0.05 as compared to mTBI; cP<0.05 as compared to mTBI+AG(50); dP<0.05 as compared to mTBI+AG(100); eP<0.05 as compared to mTBI+L-NAME(10) (One-way ANOVA followed by Tukey's test).
3.1.4. DISCUSSION

In the present study, we found that American ginseng (AG) could significantly improve cognitive functions, oxidative-nitrosative stress and neuro-inflammation induced cell death cascade associated with rat model of head trauma. On the other hand, L-NAME pretreatment potentiated the beneficial effects of AG, while L-arginine appeared to reverse its protective effects. These observations clearly suggest that the beneficial effect of AG on memory performance against head injury is dependent on the modulation of nitric oxide pathway.

In the current study, video tracking system with Ethovision software was used for assessment of cognitive functions in Morris water maze. Mild traumatic brain injury (mTBI) rats showed a significant increase in escape latency time and total distance travelled (swim path) to reach the hidden platform in Morris water maze test as compared to sham group. In the probe...
trial, time spent and frequency of appearance in target quadrant were significantly decreased in mTBI group as compared to sham group. These findings are in conformity with the previous report (Siopi et al., 2012). In the present study, chronic treatment with AG significantly improved the several cognitive parameters of Morris water maze as compared to MTBI rats. The memory restorative potentials of ginseng are in line with the latest report (Al-Hazmi et al., 2013).

Oxidative stress is one of the leading factors in the pathogenesis of traumatic head injury (Bayir et al., 2003). Generation of free radicals such as super-oxide and hydroxyl ions after head trauma are the major contributors in the pathogenesis of secondary injury cascade (Kontos and Wei, 1986). Recent report suggests a strong induction of oxidative/nitrosative damage markers in a mild traumatic brain injury model of rats (Abdul-Muneer et al., 2013). Similarly, the present study showed a significant increase in lipid peroxidation, nitrite levels and a marked decrease in reduced glutathione and catalase enzyme level in hippocampus region of head trauma rats. However, chronic treatment with ginseng significantly mitigated head trauma-mediated alterations in the levels of anti-oxidant enzymes. Earlier study has shown that ginseng as well as individual ginsenosides produced potent anti-oxidant effects (Lin et al., 2008).

Cholinergic transmission is important for learning and memory, and its alteration is critically involved in the development of cognitive impairment. Hippocampus has abundant inputs from the basal forebrain cholinergic system and acetylcholine (ACh) is known to play a major role in learning and memory (Prado et al., 2006). Acetylcholine is degraded by the enzyme acetylcholinesterase (AChE), thus terminating the physiological action of the neurotransmitter in the brain (Scremin et al., 2006). In the present study, significant increase in acetylcholinesterase activity in hippocampal brain region of mTBI treated rats was observed, which were further significantly attenuated on ginseng treatment. Earlier, Banishin and its group (Banishin et al., 1989) reported that ginsenosides facilitated cholinergic neurotransmission by increasing cholinergic metabolism in the brain. Corticosterone secretion during stressful events (via HPA activation) is known to influence cognition.
and memory processes (Roozendaal, 2002). Similarly, in the present study we found a significant increase in CORT level was observed in mTBI group as compared to the sham group. Treatment with ginseng significantly restored the serum corticosterone level which is supported by finding from Xu and its group (Xu et al., 2010).

Secondary brain injury involves several mechanisms, including the initiation oxidative stress and the release of many inflammatory cytokines (Lenzlinger et al., 2001). Neuronal inflammation is a known process which is involved in the onset of several neurodegenerative disorders, including Alzheimer's disease. Further, head injury-induced changes are known to initiate the process of apoptotic cell death by release and activation of pro-apoptotic factors such as caspases (Mazzeo et al., 2009). In the present study, a significant rise in the level of pro-inflammatory cytokine (TNF-α) and apoptotic factor (caspase-3) was observed in the brain hippocampus of mTBI treated rats, which is indicative of enhanced neuroinflammation and cell death which is possibly responsible for poor cognitive performance. Our findings are in concurrence with those reported from other laboratory (Mao et al., 2012). Treatment with ginseng significantly attenuated TNF-α and caspase-3 level in brain hippocampus of mTBI rats. These evidences suggest that neuroprotective effects of ginseng are due to its anti-inflammatory and anti-apoptotic activities (Lee et al., 2012).

Nitric oxide (NO) is an essential signaling molecule involved in various physiological functions within our body. Nitric oxide reacts with reactive oxygen species and acts as an oxidant agent (Pall, 2000). Nitric oxide can inhibit phosphorylation and glycolytic pathways by causing nitrosylation of different proteins (Zhang and Snyder, 1995). Increased expression of NOS can further initiate the process of neuro-inflammation and oxidative damage (Contestabile, 2003). Neuronal injury due to excess production of both nitric oxide and peroxynitrite has been documented in the Alzheimer's disease (Koppal et al., 1999). In the current investigation, L-NAME (a non selective inhibitor of NOS) pretreatment with sub-effective dose of ginseng potentiated the protective effects of ginseng. However, L-arginine (a nitric oxide donor) pretreatment attenuated its protective effect. This is in line with the previous
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report that L-arginine raises the concentration of NO and promotes memory formation in rats (Vanaja and Ekambaram, 2004). Earlier report suggests the inhibitory effects of various flavonoids against nitric oxide (NO) production (Marzouk et al., 2007).

The findings of the present study highlights that anti-oxidant and anti-inflammatory effects of American ginseng may have increased the endogenous defensive capacity of the brain via modulation of nitric oxide pathway to combat against oxidative stress mediated inflammatory and cell death cascade, which may have resulted in protection against neurodegeneration and cognitive loss in traumatic brain injury (Fig. 3.1.8).

Fig 3.1.8 Possible mechanism of action of ginseng and its interaction with nitric oxide modulators against mild traumatic brain injury induced cognitive deficits
CHAPTER 3.2: NEUROPROTECTIVE EFFECTS OF QUECTEIN AND ITS INTERACTION WITH MINOCYCLINE AGAINST MILD TRAUMATIC BRAIN INJURY INDUCED COGNITIVE DEFICITS

3.2.1 INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity worldwide. In addition to the complexity of the injury mechanisms, factors such as age, gender, alcohol and drug use, metabolic state, comorbidities, combined trauma, and genetics also influence the effects of an intervention following TBI (Maas et al., 2008). TBI survivors often suffer from severe cognitive ability and neurologic deficits (Prins and Hovda, 2003). It is a common neurological impairment known to trigger a variety of symptoms ranging from a simple headache to permanent cognitive dysfunction. Hippocampus is the main brain structure prominently affected in the head trauma cases (Tong et al., 2002). Since hippocampal neurons play an important role in the learning and memory functions, thus their selective vulnerability may cause a risk of memory dysfunction (Ozdemir et al., 2005). The release of oxygen species and neuroinflammatory cytokines in post-traumatic stress disorder has been well studies (Pall and Satterlee, 2001). However, the exact cellular or molecular cascade of traumatic brain injury induced cognitive loss, oxidative damage and neuroinflammation is not very clear.

Microglial cells, the resident immune cells of the brain, have a critical role of immune surveillance and host defence under physiological conditions. However, excessive microglial activation leads to increase in the central inflammatory responses in the brain injury (Morganti-Kossmann et al., 2002). Activated microglia releases proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) that have been observed in human and rodent brains following TBI (Lucas et al., 2006). Microglia and their secretions are the major contributors to the enhanced death of neurons in neurodegenerative diseases (Dheen et al., 2005). Therefore, activated microglia can in turn increase secondary injury, impair recovery, and can lead to neuronal death
after brain insult. Minocycline, a semi-synthetic, long-acting tetracycline derivative with good penetration of the blood-brain barrier, has been shown to have remarkable neuroprotective properties in models of neurodegeneration (Yong et al., 2004). Aside from its direct anti-apoptotic effect, its neuroprotective function has been well documented due to reduction of inducible nitric oxide synthase (iNOS) and neuroinflammatory cytokines expression (Dean et al., 2012). Minocycline exerts its anti-inflammatory actions by modulating microglia, immune cell activation and subsequent release of cytokines, chemokines and lipid mediators of inflammation (Stirling et al., 2004). However, the long-term effect of minocycline on neurocognition and neurobehavior alterations is not yet evaluated.

Quercetin, a dietary flavonol that is frequently found in foods, is especially abundant in onions, berries, and apples (Harwood et al., 2007). Quercetin has been acknowledged to have various beneficial effects on human health including cardiovascular protective, anticancer, antiviral, and anti-inflammatory activities (Kumar et al., 2008). In addition, several previous studies have suggested that quercetin has ameliorating effects on cognitive dysfunctions induced by various compounds, such as ethanol, d-galactose, and colchicine; in addition, quercetin is thought to alleviate cognitive deficits induced by surgical methods, such as ischemia (Kumar et al., 2008). As an antioxidant, it combats the destructive “free radical” molecules that play a part in many diseases. Quercetin also prevents the ethanol-mediated reduction of intracellular antioxidant defence systems, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Molina et al., 2003). Kawabata and its group reported that quercetin attenuates stress-induced behavioral depression through reduction in the HPA axis activation by the suppression of the CRF mRNA expression in the hypothalamus (Kawabata et al., 2010). Earlier, quercetin has also been reported to attenuate high glucose-induced expression of proinflammatory cytokines (Wu et al., 2009).
Therefore, the aim of this experiment was to investigate the neuroprotective effects of quercetin and its interaction with microglial inhibitor (minocycline) against traumatic head injury induced cognitive impairment.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Animals

Adult male Wistar rats (200–250 g) were procured from Animal House of Panacea Biotec Ltd, Lalru (Panjab). Animals were housed under standard laboratory conditions (25±2°C, 60–70% humidity) and maintained on a 12 hour natural day–night cycle, with free access to food and water. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Panjab University (IAEC/170-175/UIPS/16) and conducted according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India on the use and care of experimental animals.

3.2.2.2 Mild traumatic brain injury (mTBI)

As per section (3.1.2.2)

3.2.2.3 Drugs and treatment schedule

Quercetin was purchased from Sigma (St. Louis, MO, USA) and minocycline hydrochloride from Wyeth Ltd., Mumbai, India. TNF-α and caspase-3 ELISA kits were purchased from R&D Systems, USA. All other chemicals used for biochemical estimations were of analytical grade. The animals were randomly divided into ten experimental groups (n=5-6). First and second group was named as naïve and sham group respectively. Third group was named as mTBI (mild traumatic brain injury) group. Quercetin (20, 40 and 80 mg/kg; p.o.) were treated as group 4–6 respectively. Minocycline (25 and 50 mg/kg; p.o.) treatment was categorized as group 7 and 8 respectively. Combination of quercetin (20 and 40 mg/kg; p.o.) with minocycline (25 mg/kg; p.o.) served as group 9-10 respectively. Quercetin and minocycline were prepared in peanut oil and administered orally on the basis of body weight (5 ml/100 g). After a surgical rehabilitation period of two weeks, drugs were then administered once daily for a period of another two weeks. Pictogram of the entire protocol is as per Fig. 3.1.1.
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3.2.2.4 Behavioral Assessment

3.2.2.4.1 Morris water-maze test
As per section (3.1.2.4.1)

3.2.2.5 Serum corticosterone estimation

3.2.2.5.1 Preparation of serum
As per section (1.1.2.5.1)

3.2.2.5.2 Corticosterone assessment
As per section (1.1.2.5.2)

3.2.2.6 Biochemical assessments
As per section (3.1.2.6)

3.2.2.6.1 Estimation of oxidative stress
As per section (1.1.2.6.1)

3.2.2.6.1.1 Estimation of lipid peroxidation
As per section (1.1.2.6.1.1)

3.2.2.6.1.2 Estimation of reduced glutathione
As per section (1.1.2.6.1.2)

3.2.2.6.1.3 Estimation of superoxide dismutase
As per section (1.1.2.6.1.3)

3.2.2.6.1.4 Estimation of catalase
As per section (1.1.2.6.1.4)

3.2.2.6.1.5 Protein estimation
As per section (1.1.2.6.1.5)

3.2.2.6.2 Assessment of nitrosative stress
As per section (1.1.2.6.2)

3.2.2.7 Molecular estimations

3.2.2.7.1 Estimation of tumor necrosis factor-alpha (TNF-α)
As per section (3.1.2.7.1)
3.2.2.7.2 Estimation of apoptotic factor (caspase-3)

As per section (3.1.2.7.2)

3.2.2.8 Histopathology of brain tissue

3.2.2.8.1 Tissue sections preparation

The animals were deeply anaesthetized and perfused transcardially via the ascending aorta with cold phosphate buffered saline (0.1 M, pH 7.4) followed by fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M PBS solution (pH 7.4). The hippocampal regions of brain was dissected out and fixed overnight at 4°C in the same buffer containing 4% (w/v) paraformaldehyde. The brain parts were then washed with 0.1 M PBS (pH 7.4) for 1 h, dehydrated in alcohol, and then embedded in paraffin wax. Serial coronal sections (5 μm thickness) of brain parts were then obtained.

3.2.2.8.2 Hematoxylin and eosin (H&E) staining

The paraffin sections (thickness 5 μm) were dewaxed and rehydrated with alcohol for hematoxylin-eosin (H&E) staining. The neurons in hippocampus were examined under microscopy and photomicrographs were prepared.

3.2.2.9 Statistical analysis

As per section (3.1.2.8)

3.2.3 RESULTS

3.2.3.1 Minocycline modulates the protective effects of quercetin on escape latency time (ELT) in Morris water maze test

Traumatic brain injury significantly decreased escape latency time (ELT) to reach the hidden platform from day 24-27 as compared to the sham group (p<0.05). Sham treatment did not show any significant effect on ELT as compared to naive group. Lower dose of quercetin (20 mg/kg) and minocycline (25 mg/kg) drug treatment for 14 days did not show any significant effect on ELT as compared to control. Further, quercetin (40, 80
mg/kg) and minocycline (50 mg/kg) treatment for 14 days significantly shortened ELT as compared to mTBI control (p<0.05). Further, co-administration of sub effective doses of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) significantly potentiated their protective effects (decreased ELT) which was also significant as compared to their effects alone (p<0.05). Further, per se treatment of quercetin (80 mg/kg) did not show any significant effect on ELT as compared to sham (data not shown) (Fig. 3.2.1).

Fig. 3.2.1 Effects of quercetin and its co-administration with minocycline on escape latency time in Morris water maze test. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), quercetin (20, 40, 80 mg/kg); M, minocycline.
3.2.3.2 Minocycline modulates the protective effects of quercetin on frequency and time spent in target quadrant (TSTQ)

mTBI treated rats failed to remember the location of platform on day 28 (probe trial) and showed significantly less frequency and TSTQ as compared to sham group (p<0.01). Sham treatment did not show any significant effect on frequency (Fig. 3.2.2.1) and TSTQ (Fig. 3.2.2.2) as compared to naïve group. Quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment for 14 days did not show any significant effect on frequency and TSTQ as compared to control. Further, quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment for 14 days significantly improved frequency and TSTQ as compared to mTBI control (p<0.01). Further, treatment of sub effective dose of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days significantly potentiated their protective effect (decreased frequency and TSTQ) which was significant as compared to their effects alone (p<0.01). However, per se treatment of quercetin (80 mg/kg) did not show any significant effect as compared to sham (data not shown).

Fig. 3.2.2.1 Effects of quercetin and its co-administration with minocycline on frequency of appearance in target quadrant. Values are expressed as mean ± SEM. For statistical significance, "P <0.05 as compared to sham group; "P <0.05 as compared to mTBI; "P <0.05 as compared to mTBI+Q(20); "P <0.05 as compared to mTBI+Q(40); "P <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey's test). mTBI, mild
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traumatic brain injury; Q(20, 40, 80), Quercetin (20, 40, 80 mg/kg); M, Minocycline.

Fig. 3.2.2.2 Effects of quercetin and its co-administration with minocycline on time spent in target quadrant (TSTQ). Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), quercetin (20, 40, 80 mg/kg); M, minocycline.

3.2.3.3 Minocycline modulates the protective effects of quercetin on total distance travelled to reach the hidden platform (path length)

mTBI treated rats showed a significant increase in path length as compared to sham animals (p<0.05). Sham treatment did not show any significant effect on path length as compared to naïve group. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment significantly shortened the path length as compared to mTBI control (p<0.05). However, quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment did not show any significant effect on path length as compared to mTBI control. Further, treatment of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) significantly potentiated their protective effect which was also significant as compared their effects alone (p<0.05) (Table 3.2.1). Further, the computer tracking of the path travelled to reach the hidden platform is shown in Fig 3.2.3.
Table 3.2.1 Effects of quercetin and its co-administration with minocycline on total distance travelled to reach the hidden platform (path length)

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>2132.5±102.1</td>
<td>1331.2±134.5</td>
<td>1054.6±131.1</td>
<td>812.6±74.5</td>
</tr>
<tr>
<td>Sham</td>
<td>2145.2±124.1</td>
<td>1356.7±122.1</td>
<td>1037.8±122.4</td>
<td>824.5±82.5</td>
</tr>
<tr>
<td>mTBI</td>
<td>2203.1±122.5</td>
<td>2145.6±133.4</td>
<td>2022.4±154.8</td>
<td>1912.4±123.4</td>
</tr>
<tr>
<td>mTBI + Q(20)</td>
<td>2168.4±187.2</td>
<td>2098.3±124.5</td>
<td>1965.7±126.1</td>
<td>1856.7±132.2</td>
</tr>
<tr>
<td>mTBI + Q(40)</td>
<td>2112.4±156.5</td>
<td>1745.4±113.4</td>
<td>1645.6±126.1</td>
<td>1411.2±112.3</td>
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<td>mTBI + Q(80)</td>
<td>2145.6±134.8</td>
<td>1432.6±115.7</td>
<td>1234.6±178.9</td>
<td>1010.4±92.4</td>
</tr>
<tr>
<td>mTBI + M(25)</td>
<td>2104.4±112.3</td>
<td>2045.6±114.6</td>
<td>1932.1±113.4</td>
<td>1878.6±90.5</td>
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<td>mTBI + M(50)</td>
<td>2212.3±105.3</td>
<td>1511.4±124.6</td>
<td>1332.5±123.4</td>
<td>1095.6±122.4</td>
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<tr>
<td>mTBI + Q(20) + M(25)</td>
<td>2234.2±145.3</td>
<td>1758.5±131.9</td>
<td>1684.6±161.2</td>
<td>1499.4±128.8</td>
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<td>2215.1±114.3</td>
<td>1489.4±124.5</td>
<td>1251.6±132.1</td>
<td>1021.1±112.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, P <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), Quercetin (20, 40, 80 mg/kg); M, Minocycline.
3.2.3.4 Minocycline modulates the protective effects of quercetin on serum corticosterone (CORT)

Traumatic brain injury caused a significant rise in serum CORT level as compared to sham group (p<0.01). Sham treatment did not show any significant effect on serum CORT level as compared to naive group. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment for 14 days significantly attenuated the serum CORT level as compared to mTBI control. Further, quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment for 14 days did not show any significant effect on serum CORT level as compared to mTBI control. However, treatment of sub effective doses of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days significantly potentiated their protective effect (attenuated serum CORT level) which was also significant as compared to their effects alone (p<0.01). Further, per se treatment of
quercetin (80 mg/kg) did not show any significant effect on serum CORT level as compared to sham (data not shown) (Fig. 3.2.4).

**Fig. 3.2.4. Effects of quercetin and its co-administration with minocycline on serum corticosterone (CORT).** Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test).

### 3.2.3.5 Minocycline modulates the protective effects of quercetin on oxidative-nitrosative stress

mTBI treatment significantly increased oxidative and nitrosative stress as compared to sham (p<0.05). Sham treatment did not show any significant effect as compared to naïve group. However, treatment with quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) for 14 days significantly attenuated the oxidative-nitrosative damage as compared to mTBI control. Quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment did not show any significant effect as compared to control. Further, treatment of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days significantly potentiated their effects (attenuated oxidative-nitrosative stress) which was also significant as compared to their effects alone (p<0.05). Also, per se treatment of quercetin (80 mg/kg) did not show any significant effect on oxidative-nitrosative stress as compared to sham (data not shown) (Table 3.2.2).
Table 3.2.2 Effects of quercetin and its co-administration with minocycline on oxidative-nitrosative stress

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LPO (mole of MDA/mg pr (% of sham))</th>
<th>GSH (µmole of GSH/mg pr (% of sham))</th>
<th>SOD (units/mg pr (% of sham))</th>
<th>Catalase (µmole of H₂O₂/min/mg pr (% of sham))</th>
<th>Nitrite (µg/ml (% of sham))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0.105±0.02 (96.3)</td>
<td>0.058±0.003 (95.1)</td>
<td>1.26±0.25 (103.3)</td>
<td>0.61±0.01 (101.7)</td>
<td>232.4±9.4 (92.4)</td>
</tr>
<tr>
<td>Sham</td>
<td>0.109±0.03 (100)</td>
<td>0.061±0.003 (100)</td>
<td>1.22±0.44 (100)</td>
<td>0.60±0.03 (100)</td>
<td>251.5±11.2 (100)</td>
</tr>
<tr>
<td>mTBI</td>
<td>0.435±0.04a (399.1)</td>
<td>0.012±0.003a (20.7)</td>
<td>0.23±0.02a (18.9)</td>
<td>0.11±0.01a (18.3)</td>
<td>564.8±15.1a (224.7)</td>
</tr>
<tr>
<td>mTBI + Q(20)</td>
<td>0.411±0.05 (377.1)</td>
<td>0.018±0.002 (29.5)</td>
<td>0.31±0.05 (25.4)</td>
<td>0.16±0.03 (26.7)</td>
<td>541.5±15.3 (215.5)</td>
</tr>
<tr>
<td>mTBI + Q(40)</td>
<td>0.313±0.05b,c (287.2)</td>
<td>0.031±0.003b,c (50.8)</td>
<td>0.72±0.04b,c (59.1)</td>
<td>0.33±0.03b,c (55)</td>
<td>432.6±12.4b,c (172.1)</td>
</tr>
<tr>
<td>mTBI + Q(80)</td>
<td>0.184±0.03c,d (168.8)</td>
<td>0.051±0.004c,d (83.6)</td>
<td>1.12±0.03c,d (91.8)</td>
<td>0.52±0.03c,d (86.7)</td>
<td>307.4±13.4c,d (122.3)</td>
</tr>
<tr>
<td>mTBI + M(25)</td>
<td>0.387±0.08 (355.1)</td>
<td>0.023±0.007 (37.7)</td>
<td>0.35±0.04 (28.7)</td>
<td>0.19±0.04 (31.7)</td>
<td>524.6±12.3 (208.8)</td>
</tr>
<tr>
<td>mTBI + M(50)</td>
<td>0.213±0.05b,e (195.4)</td>
<td>0.040±0.006b,e (65.6)</td>
<td>1.08±0.03b,e (88.5)</td>
<td>0.50±0.05b,e (83.3)</td>
<td>324.5±14.8b,e (129.1)</td>
</tr>
<tr>
<td>mTBI+Q(20)+ M(25)</td>
<td>0.320±0.05c,e (293.8)</td>
<td>0.032±0.006c,e (52.5)</td>
<td>0.68±0.10c,e (55.7)</td>
<td>0.30±0.05c,e (50)</td>
<td>425.7±14.2c,e (169.3)</td>
</tr>
<tr>
<td>mTBI+Q(40)+ M(25)</td>
<td>0.192±0.03d,e (176.1)</td>
<td>0.049±0.006d,e (80.3)</td>
<td>1.06±0.06d,e (86.9)</td>
<td>0.49±0.05d,e (81.7)</td>
<td>312.4±10.5d,e (124.3)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), Quercetin (20, 40, 80 mg/kg); M, Minocycline.
3.2.3.6 Minocycline modulates the protective effects of quercetin on acetylcholinesterase (AChE) activity

mTBI significantly increased AChE activity in hippocampus as compared to sham group (p<0.05). Sham treatment did not show any significant effect on AChE activity as compared to naive group. However, treatment with quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) for 14 days significantly attenuated AChE activity as compared to mTBI control. Further, lower doses of quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment did not show any significant effect on AChE activity as compared to mTBI control. Co-administration of sub effective doses of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days significantly attenuated AChE activity which was also significant as compared to their effects alone (p<0.05). Further, per se treatment of quercetin (80 mg/kg) did not show any significant effect on AChE activity as compared to sham (data not shown) (Fig. 3.2.5).

![Fig. 3.2.5 Effects of quercetin and its co-administration with minocycline on AChE activity. Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), Quercetin (20, 40, 80 mg/kg); M, Minocycline.](image-url)
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3.2.3.7 Minocycline modulates the protective effects of quercetin on tumor necrosis factor (TNF-α) and apoptotic factor (caspase-3)

There was a significant increase in hippocampal TNF-α and caspase-3 level in mTBI treated animals as compared to sham group (p<0.05). Sham treatment did not show any significant effect on TNF-α and caspase-3 level as compared to naive group. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment significantly attenuated TNF-α (Fig. 3.2.6.1) and caspase-3 (Fig. 3.2.6.2) level as compared to control. Further, quercetin (20 mg/kg) and minocycline (25 mg/kg) treatment did not show any significant effect on TNF-α and caspase-3 level as compared to control. Co-administration of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days significantly potentiated their protective effect (attenuated TNF-α and caspase-3 level) which was also significant as compared to their effects alone (p<0.05). Also, per se treatment of quercetin (80 mg/kg) did not show any significant effect on TNF-α and caspase-3 level as compared to sham (data not shown).

Fig. 3.2.6.1 Effects of quercetin and its co-administration with minocycline on tumor necrosis factor (TNF-α). Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test).
Fig. 3.2.6.2 Effects of quercetin and its co-administration with minocycline on apoptotic factor (caspase-3). Values are expressed as mean ± SEM. For statistical significance, aP <0.05 as compared to sham group; bP <0.05 as compared to mTBI; cP <0.05 as compared to mTBI+Q(20); dP <0.05 as compared to mTBI+Q(40); eP <0.05 as compared to mTBI+M(25) (One-way ANOVA followed by Tukey’s test). mTBI, mild traumatic brain injury; Q(20, 40, 80), quercetin (20, 40, 80 mg/kg); M, minocycline.

3.2.3.8 Minocycline modulates the protective effects of quercetin on histopathological alterations in brain hippocampus

Brain traumatic injury caused a marked increase in the numbers of neuroinflammatory as well as apoptotic cells in brain hippocampal region as compared to sham. Sham treatment did not show much effect as compared to naive (data not shown). However, treatment with quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) for 14 days attenuated these histopathological alterations and resulted in reduced neuro-inflammatory cells and apoptosis. Further, quercetin (20 mg/kg) and minocycline (25 mg/kg) for 14 days did not show any effect on histopathology as compared to mTBI control (data not shown). Further, treatment of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) for 14 days further potentiated their protective effects (attenuated the neuroinflammatory and apoptotic cells) as compared to their effects per se (Fig. 3.2.7).
Fig. 3.2.7 Effects of quercetin and its co-administration with minocycline on brain histopathology.

Sections were stained with Haematoxylin and Eosin (HE stain × 250). Black arrows indicate neuroinflammatory and apoptotic cells. 1: Sham: neurons are intact. 2: mTBI control: severe inflammation and apoptosis. 3: mTBI+ Q (40 mg/kg): mild inflammation of neurons with fewer apoptotic cells. 4: mTBI+ Q (80 mg/kg): reduction of inflammatory neurons. 5: mTBI+ Q (20 mg/kg) + M (25 mg/kg): mild inflammation of neurons with apoptotic cells. 6: mTBI+ Q (40 mg/kg) + M (25 mg/kg): Diminished amount of neuroinflammation (Scale bar-50μm).

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3.3.4 DISCUSSION

Memory impairment is a common consequence of head trauma, observed in various age groups particularly among young population (Ozdemir et al., 2012). Damage to the cognitive cores of the brain can evoke stress-related responses, and results into permanent memory loss (Belujon and Grace, 2011). In the present study, quercetin treatment attenuated cognitive deficits, oxidative stress, neuro-inflammation and apoptosis suggesting its neuroprotective effects against rat model of head trauma. Besides, neuroprotective effects of quercetin were potentiated by treatment with minocycline, a known microglia inhibitor suggesting the role of microglia inhibition in its neuroprotective effects.

There was a significant increase in escape latency time and total distance travelled (swim path) to reach the hidden platform in Morris water maze test in case of mild traumatic brain injury (mTBI) rats as compared to sham group. Further, time spent and frequency of appearance in target quadrant in probe trial was significantly decreased in mTBI group as compared to sham animals. These observations are in line with the previous findings which suggest that head injury leads to spatial behavioral deficits (Henninger et al., 2005). Treatment with quercetin and minocycline significantly improved the cognitive performance in Morris water maze as compared to mTBI rats. Further, quercetin treatment with minocycline potentiated their protective effects, suggesting the role of microglial inhibitory pathway. The results are in line with the evidence which suggests that quercetin improves learning and memory performance in experimental animals (Kumar et al., 2008). Minocycline, a derivative of tetracycline having an ability to cross blood brain barrier (BBB), has its neuroprotective effect in neurological conditions, such as including animal models of cerebral ischemia, Huntington’s disease and Alzheimer’s disease (Parachikova et al., 2010).

Apart from behavioral modifications, neurotransmitter alterations such as changes acetylcholine (ACh) levels also results in the development of cognitive deficits (Prado et al., 2006). Acetylcholine is degraded by the
enzyme acetylcholinesterase (AChE), thus terminating its physiological effect in the brain. Traumatic brain injury results in loss of cholinergic neurons and alteration of the acetylcholine (ACh) neurotransmission in adult animals (Scremin et al., 2006). In the present study, there was a significant increase in acetylcholinesterase activity in brain hippocampal region of head trauma rats which was later significantly attenuated by treatment with quercetin, minocycline and their combination. Minocycline has been shown to exhibit long-term neuroprotective effects against cognitive impairment (Jin et al., 2013). These results are in line with the earlier findings (Pachauri et al., 2012). Further, the secretion of glucocorticoids during stressful events leads to HPA axis activation and further influence retention and retrieval of memory (Roozendaal, 2002). Supporting to these reports, we found a significant increase in serum corticosterone level in mTBI animals as compared to the naive animals. Earlier study has documented that quercetin attenuated corticotrophin releasing factor (CRF) induced anxiogenic and depressant-like effects by normalization of HPA axis activity (Bhutada et al., 2010a). Similarly, in the present study treatment with quercetin and minocycline significantly reduced the corticosterone level associated with head injury. Further, quercetin treatment and its combination with minocycline potentiated their protective effects, suggesting the involvement of microglial inhibition pathway.

It is well known that oxidative stress is an important contributor in the pathogenesis of traumatic head injury (Bayir et al., 2003). The secondary brain injury involves several mechanisms, including the release of immune mediators like interleukins and the initiation of acute oxidative stress (Lenzlinger et al., 2001). Kontos and Wei were the first to demonstrate the formation of superoxide radicals in the cerebral microvasculature in a fluid-percussion brain injury model and (Kontos and Wei, 1986). Head trauma induced super-oxide and hydroxyl ions are thought to play a major role in the progress of secondary injury cascades (Kontos and Wei, 1986). Recent study also suggests involvement of oxidative damage in experimental model of traumatic brain injury (Abdul-Muneer et al., 2013). Supporting the above reports, the present study also showed a significant increase in oxidative stress markers i.e. increase in lipid peroxidation, nitrite levels and a marked
decrease in reduced glutathione, superoxide dismutase and catalase enzyme level in hippocampus of head trauma rats. In the present study, treatment with quercetin and minocycline significantly attenuated oxidative-nitrosative stress markers. Further, quercetin treatment with minocycline potentiated their protective effects, suggesting the role of microglial inhibition pathway in their neuroprotective effects. Minocycline and other microglial inhibitors have also been demonstrated to exert their anti-oxidative like effect as a direct scavenger of free radicals which is responsible for the neuroprotective effect (Kraus et al., 2005).

Neuroinflammation has been well known mediator of secondary injury in moderate and severe head injury (Maas et al., 2008). While primary injury leads to edema formation and tissue loss within the brain, secondary injury involves cellular cascades, including release of many pro-inflammatory cytokines (Lenzlinger et al., 2001). Johnson and its group showed that chronic neuroinflammatory changes are present in the white matter of the brain and relate to progressive changes in white matter integrity following traumatic brain injury (Johnson et al., 2013). Pro-inflammatory cytokine, such as TNF-α is produced by microglial cells, astrocytes, neutrophils and macrophages and augment both inflammation and subsequent immune responses. Recently it has been suggested that increase in inflammatory mediators after traumatic brain injury may contribute to progressive neurodegeneration and release of many apoptotic factors (Schaible et al. 2013), and finally results into cognitive deficits. Similarly, in the present study, we observed a significant elevation of pro-inflammatory cytokines (TNF-α) and apoptotic factor (caspase-3) levels in hippocampus areas of brain trauma rats which is indicative of enhanced neuroinflammation and neuronal death. Further, treatment with quercetin, minocycline and their combination significantly attenuated level of pro-inflammatory cytokine and apoptotic factor in head trauma rats.

Further, co-administration of subeffective doses of quercetin and minocycline potentiated their protective effects, suggesting the involvement of common microglial inhibition pathway. Earlier, quercetin is known to block the inflammation-mediated apoptotic cell death cascade (Bureau et al., 2008). According to Kovesdi and its group, minocycline treatment normalized tissue
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levels of the majority of the inflammatory and glial markers in an experimental model of brain traumatic injury (Kovesdi et al., 2012). Earlier, it has been reported that minocycline treatment suppressed microglial production of inflammatory cytokines as well as amyloid precursor protein (APP) in transgenic mice (Seabrook et al., 2006). All these evidences possibly suggest that quercetin modulated the microglial inhibitory effects of minocycline and blocked the neuroinflammation and apoptotic pathway in rat model of head injury. These results are further evidenced by histopathological studies.

Taken together, the present study suggests the neuroprotective effects of quercetin that could be possibly due to its microglial inhibitory pathway in attenuating neuroinflammation and cognitive deficits against traumatic brain injury (Fig. 3.2.8).

Fig 3.2.8 Possible mechanism of action of quercetin and its interaction with minocycline against mild traumatic brain injury induced cognitive loss

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