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RESTORATION OF GLUTAMINE SYNTHETASE ACTIVITY, NITRIC OXIDE LEVELS AND AMELIORATION OF OXIDATIVE STRESS BY PROPOLIS IN KAINIC ACID MEDIATED EXCITOTOXICITY

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Abstract

Background: Propolis has been proposed to be protective on neurodegenerative disorders. To understand the neuroprotective effects of honeybee propolis, glutamine synthetase (GS) activity, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS) and total antioxidant status (TAS) were studied in different brain regions- cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of rats supplemented with propolis and subjected to kainic acid (KA) mediated excitotoxicity.
Materials and Methods: Male *Sprague-Dawley* rats were divided into four groups; Control group and KA group received vehicle and saline. Propolis group and propolis + KA group were orally administered with propolis (150mg/kg body weight), five times every 12 hours. KA group and propolis + KA group were injected subcutaneously with kainic acid (15mg/kg body weight) and were sacrificed after 2 hrs and CC, CB and BS were separated homogenized and used for estimation of GS activity, NO, TBARS, and TAS concentrations by colorimetric methods. Results were analyzed by one-way ANOVA, reported as mean $\pm$ SD from 6 animals, and $p<0.05$ considered statistically significant.
Results: NO was increased ($p< 0.001$) and GS activity was decreased ($p< 0.001$) in KA treated group compared to control group as well as propolis + KA treated group. TBARS was decreased and TAS was increased ($p< 0.001$) in propolis + KA treated group compared KA treated group.
Conclusion: This study clearly demonstrated the restoration of GS activity, NO levels and decreased oxidative stress by propolis in kainic acid mediated excitotoxicity. Hence the propolis can be a possible potential candidate (protective agent) against excitotoxicity and neurodegenerative disorders.

Keywords: Nitric oxide, Glutamine Synthetase, Oxidative Stress, Excitotoxicity, Propolis, Rat Brain.

Introduction

Glutamate and related excitatory amino acids are considered as major neurotransmitters in the central nervous system (CNS) and in that they are released by an estimated 40% of all synapses (Coyle and Puttafarcken, 1993). In addition to their ability to transmit vital excitatory CNS signals, they have shown to cause neuronal dysfunction by over stimulation of neurons. The ensuing excitotoxicity may be a causative factor in multitude of neurodegenerative diseases (Dawson et al., 1995; Dong et al., 2009). Astrocytes play a crucial role in regulating and maintaining the extracellular chemical milieu of the central nervous system under physiological conditions (Eid et al., 2013). In, the conversion of glutamate to glutamine by glutamine synthetase, that takes place within the astrocytes, represents a key mechanism in the regulation of excitatory neurotransmission under normal conditions as well as in injured brain (Szatkowski and Attwell, 1994). Thus GS is involved in modulation of the turnover of glutamate through the glutamate-glutamine cycle (Van der berg and Garfinkel, 1971). The known stoichiometry of glutamate transport across the astrocyte plasma membrane also suggests that rapid metabolism of intracellular glutamate via glutamine synthetase (GS) is a prerequisite for efficient glutamate clearance from the extracellular space (Eid et al., 2013).

Kainic acid (KA) is a potent CNS excitotoxin producing an acute and sub-acute epilepticform activity, ultimately resulting in wide spread irreversible neuropathological changes (Sperk, 1994). KA induced status epilepticus was associated with both apoptotic and necrotic cell death and induction of heat sensitive proteins in hippocampus and cortical regions of rodent brain (Akbar et al., 2001; Kato et al., 1999; White, 2002). The exact mechanisms contributing to increased concentration of nitric oxide (NO) in epilepsy are not well established. Earlier studies reported that nitric oxide synthase (NOS) knockout mice were more severely affected by epileptic activity than controls and the response to NO during epilepsy depends on its concentration (Itoh and Watanabe, 2009). It was also indicated that NO may be regarded as an anticonvulsant and proconvulsant substance in relation to convulsions induced by pentylenetetrazole (PTZ)(Itoh and Watanabe, 2009). Reactive Oxygen Species (ROS)/Reactive Nitrogen Species (RNS) have been implicated in the pathogenesis of various neurological disorders including epilepsy (Frantseva et al., 2000). Intracellular ROS are capable of inducing damage and, in severe cases, cell death through mitochondrial alterations leading to the release of cytochrome c (Berman and Hastings, 1999; Halestrap et al., 2000), through activation of the JNK pathway (Tournier et al., 2000) or by activation of nuclear factor-$_{K}$B (NF-$_{K}$B) transcription factors (Luo et al., 1999). The ability to control ROS is thus critical in neurodegenerative diseases, because neuronal damage occurs when the "oxidant- anti-oxidant" balances are disturbed in favor of excess oxidative stress (Maalouf et al., 2007). Stimulation of glutamate-KA receptors induces neuronal NO release, which in turn modulates glutamate transmission (Alabadi et al., 1999; Nakaki etal., 2000). NO induces changes in neuronal and signaling-related functions by several ways (Prast and Philippu, 2001).

Honey bee propolis has been widely used as a folk medicine and proposed to be protective on neurodegenerative disorders (Ha et al., 2010; Kwon et al., 2004). It has been shown to have broad biological activities, which are principally attributed to the presence of flavonoids (Isla et al., 2001) and caffeic acid phenyl ester (CAPE) (Natarajan et al., 1996). The prevailing opinion is that the broad biological activities of flavonoids and CAPE are related, in part, to their anti-inflammatory and anti oxidant actions (Isla et al., 2001; Natarajan et al., 1996). It was earlier reported that GS becomes nitrated and inhibited during PTZ induced seizure model at repeated PTZ seizure induction, but there was no decrease in GS protein level (Bidmon et al., 2008). Our earlier studies demonstrated that increased production of NO, increased activity of NOS, decreased activity of GS and increased oxidative stress in KA mediated excitotoxicity (Swamy et al., 2009, 2011a). Therefore the present study was conducted to assess the neuroprotective effects of the bee product propolis , by estimating the glutamine synthetase activity, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS) concentration and total antioxidant status (TAS) in cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of rats supplemented with propolis and subjected to KA mediated excitotoxicity.

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Material and Methods

Propolis collection and ethanol extraction

Honey bee propolis was obtained from local bee products shop and it was subjected to 80% ethanol extract as per the procedure described by Isla et al. (2001).

Animals

Male *Sprague Dawley* rats weighing 200 – 250 grams were used for the study. The animals had free access to food and water. They were fed with commercial feed and had access to water *ad libitum.* They were housed under standard condition of constant temperature; humidity and a 12h light/dark cycle were maintained. Animal handling and experimental design was approved by the Animal ethics committee of Universiti Sains Malaysia, Health campus, Kubang Kerian, Malaysia [USM / Animal Ethics Approval / 20011 / (68) (296)].

Experimental Study

The rats were divided in to one control group and three study groups; KA group, propolis group and propolis + KA group. Control group and KA group received vehicle and saline. Propolis group and propolis + KA were orally administered with ethanol-extracted propolis (150mg/kg body weight), five times every 12 hours as described by Kwon et al. (2004). KA group and propolis + KA group rats were given subcutaneous injection of kainic acid (15mg/kg body weight) (Milatovic et al., 2002) and were sacrificed after 2hrs of KA injection. Control group and propolis group rats were given normal saline and sacrificed after 2hrs of saline injection.

After the rats sacrificed by decapitation the brain regions –CC, CB, and BS were separated according to the procedure described by Sadasivudu and Lajtha (1970). Each of the brain regions was weighed and used for the preparation of homogenates in 0.05M phosphate buffer pH 7.3.

Enzyme assay

GS activity was assayed by the method Rowe et al. (1970) as described by Swamy et al (2011a).

Estimations of NO, TBARS and TAS:

NO was estimated as NOx (Nitrate/Nitrite) by Griess reaction after conversion of nitrate to nitrite by nitrate reductase, as described by Swamy et al (2011a) using the commercially available Nitric Oxide Assay Kit from Cayman Chemical Company (Catalogue number 780001; Ann Arbor, Michigan, USA). Lipid per oxidation was determined by the method of Chattered et al. (2000) by estimating TBARS as described by Swamy et al (2011a). TAS was estimated according to the method of Koracevic et al (2000) as described by Swamy et al (2011a).

Statistical analysis

Results were reported as mean $\pm$ standard deviation (SD) from 6 animals for each parameter calculated. Statistical analysis of results was done by one-way analysis of variance (ANOVA) followed by post hoc analysis using Bonferroni's test, using the SPSS software (version 20) to determine the statistical significance of difference in values between the control and study groups. p value of < 0.05 was taken as statistically significant at 95% confidence interval.

Results

The concentration of NO was increased significantly ($p<0.001$) in all the three brain regions tested in KA group compared to control group, but the increase of NO concentration by KA was prevented by prior supplementation of propolis. There was no significant difference in NO level between control and propolis as well as propolis + KA group (Figure 1).

GS activity was decreased significantly ($p<0.001$) in all the three brain regions in KA group compared to control group and propolis + KA group indicating propolis treatment was preventing ($p<0.001$ in CB; $p<0.01$ in CC and BS) the GS activity decrease observed by KA treatment. There was no significant difference in GS activity between control and propolis as well as propolis + KA group propolis + KA group (Figure 2).

The concentration of TBARS was increased significantly ($p<0.001$) in all the three brain regions tested in KA group compared to control group, but the increase of TBARS concentration by KA was prevented ($p<0.001$) by prior supplementation with propolis (propolis + KA group). There was no significant difference in TBARS concentration between control and propolis as well as propolis + KA group (Figure 3).

The concentration of TAS was decreased significantly ($p<0.001$) in KA group compared to control and propolis + KA group indicating the depletion of TAS concentration by KA was prevented ($p<0.001$ in CB; $p<0.01$ in CC and BS) by supplementation of propolis (propolis + KA group).

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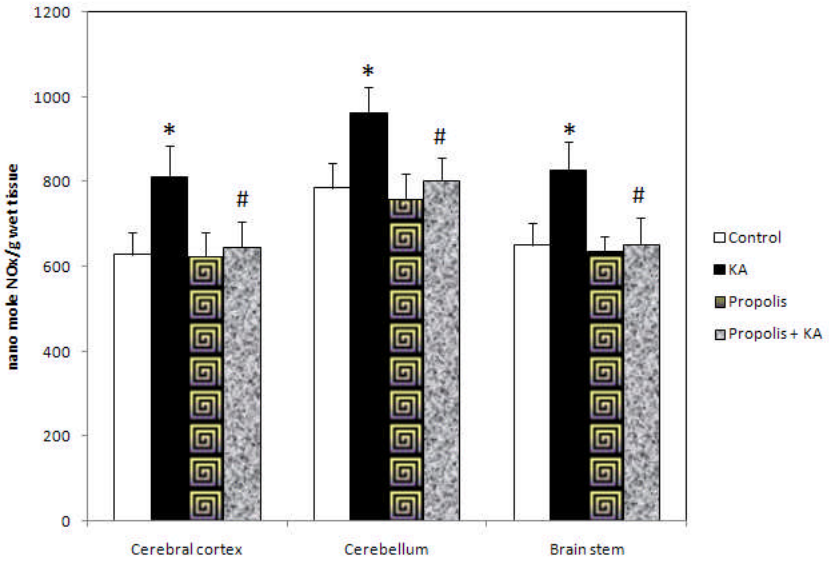
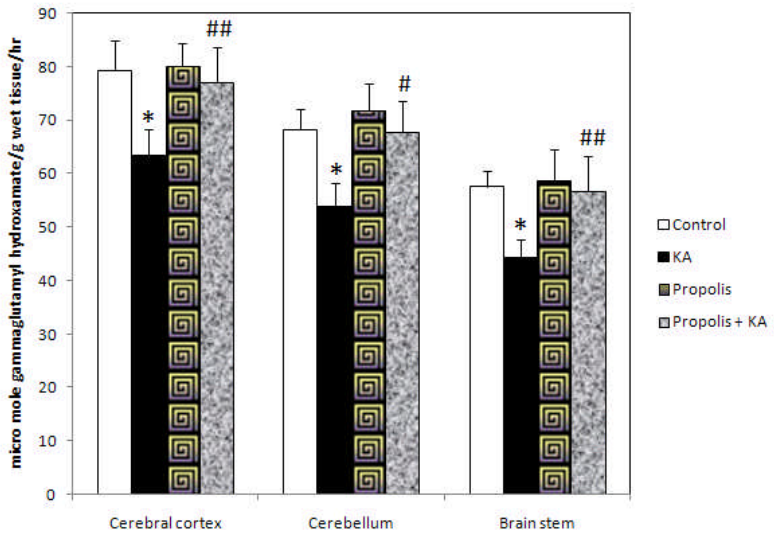
There was no significant difference in TAS concentration between control and propolis as well as propolis + KA group propolis + KA group (Figure 4).



Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001 versus KA group

Figure 1: Effect of propolis on concentration of NO in KA mediated excitotoxicity



Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001, ##p<0.01 versus KA group

Figure 2: Effect of propolis on activity of GS in KA mediated excitotoxicity

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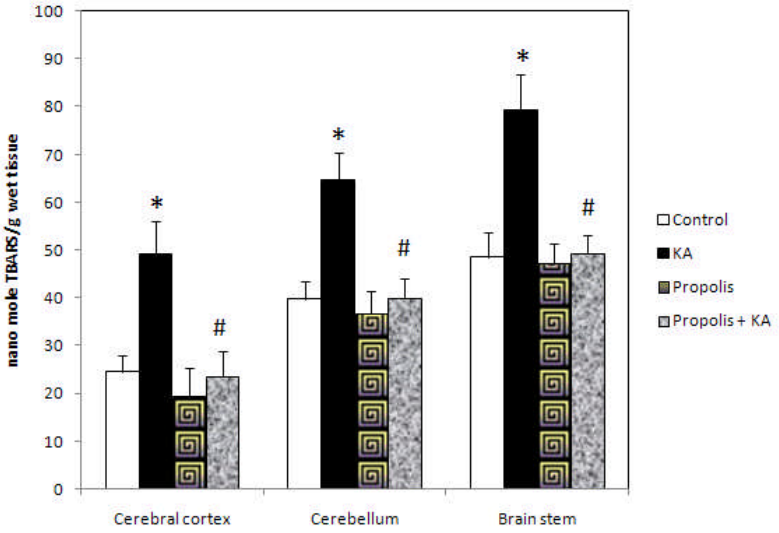
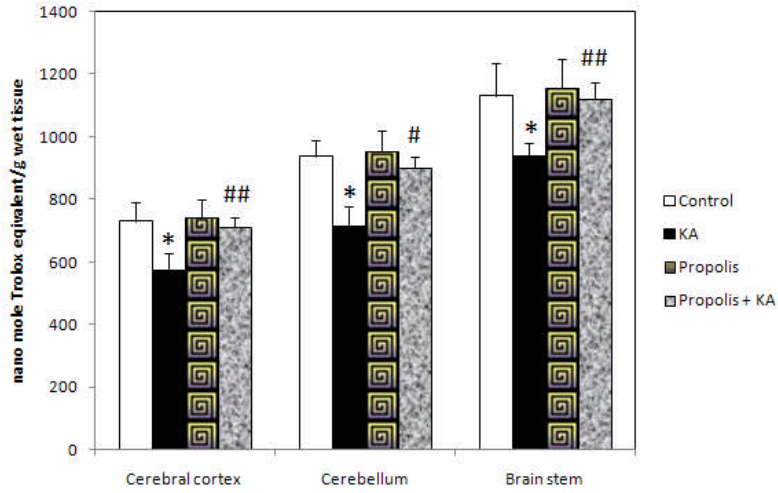


Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001 versus KA group

Figure 3: Effect of propolis on concentration of TBARS in KA mediated excitotoxicity



Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001, ##p<0.01 versus KA group

Figure 4: Effect of propolis on concentration of TAS in KA mediated excitotoxicity

Discussion

Glutamate, an excitatory amino acid, is one of the major neurotransmitter in the CNS. Evidences are shown that glutamate is highly neurotoxic when accumulated in high amount in the extra cellular space (Rothman and Olney, 1986; Takahashi et al., 1997). In the brain, the conversion of glutamate to glutamine by GS, that takes place within the astrocytes, represents a key mechanism in the regulation of excitatory neurotransmission (Szatkowski and Attwell, 1994). The glutamine synthetase activity is present in all parts of brain and it is equally high in cerebral cortex, cerebellum and hippocampus (Girard et al., 1993; Rose and Felipo 2005). The modulation of GS activity in brain, therefore, is important and its impairment or saturation may have pathological consequences (Rodrigo and Felipo 2007). Neuronal excitation involving the excitatory glutamate receptors is recognized as an important underlying mechanism in neurodegenerative disorders (Wang et al. 2005). Several studies have indicated that the activity of GS in astrocytes is diminished in several brain disorders, including epilepsy (Eid et al., 2012). Earlier studies have shown the decreased activity and expression of GS in kainic acid induced epilepsy and it has been hypothesized that the loss of GS activity in epilepsy leads to increased extracellular glutamate concentrations and epileptic seizures (Swamy et al., 2011a, b).

In neurons, NO synthesis is stimulated by Ca^{2+}-influx, which is induced by activation of glutamate receptors, preferentially NMDA receptor (Radenovic and Selakovic, 2005). NO is known to be involved in the pathophysiology of many epilepsy models resulting from increased action of

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excitatory neurotransmitter namely glutamate (Lapouble et al., 2002; Penix et al., 1994; Rundfeldt et al., 1995). The literature findings implicate neuronal NO generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries *in vivo*. Although NMDA receptors likely contribute critically to neuronal injury in various acute conditions, several observations support the hypothesis that AMPA/KA receptors may be of greater importance to the neurodegenerative process (Carriedo et al., 1998, 2000).

Excitotoxicity and disrupted energy metabolism were considered to be acting in a synergistic manner leading to nerve cell death in neurodegenerative disorders (Dong et al., 2009). These cooperative pathways trigger oxidative stress by free radical formation (Silva-Adaya et al., 2008) and ROS/RNS are believed to cause lipid per oxidation with high levels of MDA resulting in damage to biological membranes (Chan, 2001). Epileptic form activity was shown to cause excessive production of ROS/RNS, a factor believed to be involved in the mechanisms leading to neurodegeneration and cell death (Itoh and Watanabe, 2009). The increased production of NO and increased oxidative stress in kainic acid mediated excitotoxicity and epilepsy has been reported earlier (Swamy et al., 2009, 2011a).

Propolis has been used to maintain health. Pharmacological activities such as anticancer, anti inflammatory, antibiotic, ant oxidative, antifungal, anesthetic and cytostatic have been ascribed to ethanolic extracts of propolis (Isla et al., 2001). Propolis has been shown to have broad biological activities, which are principally attributed to the presence of flavonoids (major component; rutin, quercetin, galangin, etc.), phenolic compounds and CAPE (Isla et al., 2001). The beneficial actions of propolis contents namely flavonoids, phenolic compounds and CAPE are related, in part, to their anti- inflammatory and anti oxidant actions (Isla et al., 2001; Kwon et al., 2004).

It has been reported that anti- inflammatory substances lucidone (Senthil Kumar et al., 2010), Curcumin (Jung et al., 2006), and phenantroindolizdine alkaloids (Yang et al., 2006) reduce NO production observed in inflammation. Though the active ingredients involved and mechanism is not known, the supplementation of propolis in this study showed the reduced production of NO in KA mediated excitotoxicity and may be attributed to anti- inflammatory and anti oxidant action of propolis. The decreased activity of GS in excitotoxicity attributed possible modulation by high concentration of NO was shown to be abolished by supplementation of propolis in this study. The results of the study clearly indicate that the supplementation of propolis shown the amelioration of oxidative stress caused by kainic acid in all the brain regions. Hence the supplementation of propolis may be beneficial to counteract the possible ways of excitotoxicity observed in many neurological disorders.

Conclusion

Results of this study clearly demonstrated the restoration of GS activity and NO levels along with decreased oxidative stress by propolis in kainic acid mediated excitotoxicity. Hence the propolis can be a possible potential candidate (protective agent) against excitotoxicity and neurodegenerative disorders.

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