Increased nitric oxide (NO), neuronal inflammation and apoptosis have been proposed to be involved in excitotoxicity plays a part in many neurodegenerative diseases. To understand the neuro-protective effects of propolis, activities of Nitric oxide synthase (NOS) and caspase-3 along with NO and tumor necrosis factor-α (TNF-α) levels were studied in cerebral cortex (CC), cerebellum (CB) and brain stem (BS) in rats supplemented with propolis prior to excitotoxic injury with kainic acid (KA).

Materials and methods: Male Sprague-Dawley rats were divided into four groups (n=6 rats per group) as Control, KA, Propolis and KA+Propolis. The control group and KA group have 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. CC, CB and BS were separated, homogenized and used for estimation of NOS, caspase-3, NO and TNF-α by commercial kits. Results were analyzed by one way ANOVA, reported as mean ± SD (n=6 rats), and p<0.05 was considered statistically significant.

Results: The concentration of NO, TNF-α, NOS and caspase-3 activity were increased significantly (p<0.001) in all the three brain regions tested in KA group compared to the control. Propolis supplementation significantly (p<0.001) prevented the increase in NOS, NO, TNF-α and caspase-3 due to KA.

Conclusion: Results of this study clearly demonstrated that the propolis supplementation attenuated the NOS, caspase-3 activities, NO, and TNF-α concentration and in KA mediated excitotoxicity. Hence propolis can be a possible potential protective agent against excitotoxicity and neurodegenerative disorders.

Keywords: Nitric oxide; TNF-α; Caspase-3; Excitotoxicity; Propolis; Rat Brain

Introduction

The exact mechanisms involved in onset and progression of neurodegenerative diseases are still poorly defined, but excitotoxicity is recognized as one of the mechanisms involved (Atlante et al., 2001; Mattson, 203). Excessive neuronal excitation involving the excitatory glutamate receptors is recognized as an important underlying mechanism in neurodegenerative disorders. However, the exact mechanism of how excitotoxicity is implicated in neurodegeneration still needs further investigation. Kainic acid (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 excitotoxicity are not well established. Earlier studies reported that nitric oxide synthase (NOS) knockout mice were more severely affected by epileptic activity than the controls and that 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).

Excitotoxicity is commonly induced in experimental animals by KA, a 30-fold more potent glutamate agonist (Sperl, 1994). Effects of KA are mediated via activation of the kainite receptors that respond to the neurotransmitter glutamate and include the induction of inflammatory responses, production of cytokines and neuronal death (Ullah et al., 2014). The molecular mechanisms by which KA induces excitotoxicity and cell death remain unclear; however, oxidative stress and the activation of proinflammatory cytokines are major contributors (Wong et al., 2005). The cytokines and other inflammatory molecules secreted by activated glia cells can modify the outcome of disease progression. Thus, anti-oxidant and anti-inflammatory treatment could attenuate or prevent KA-induced neurodegeneration (Zhang and Zhu, 2011). Additionally, KA increases neuronal excitability, production of reactive oxygen species (ROS), and lipid peroxidation (Hasegawa et al 2008; Bruce and Baudry, 1995, Sun et al., 1992). Microglial activation and astrocytes proliferation are the other characteristics of KA-induced neurodegeneration. Both in vitro and in vivo studies demonstrate that KA induces cell death via accumulation of intracellular calcium, which stimulates ROS production and mitochondrial dysfunction, thereby leading to neuronal cell death (Wong et al., 2005; Hilton et al., 2005). There is evidence for activation of calcineurin- and caspases-induced neural apoptosis following KA exposure (Smialowska et al., 2011). Besides oxidative stress and intracellular calcium overload, KA can activate molecular mechanisms leading to depletions of neuronal energy stores and thereby activating the alternative cell death pathways (Choi, 1987; Culmsee et al., 2001; McCullough, 2005).

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). Recent studies shown that propolis supplementation reduced the oxidative stress and nitric oxide levels in KA-mediated excitotoxicity (Swamy et al., 2014). Therefore the present study was conducted to assess the neuroprotective effects of the bee product propolis, by estimating the concentration of TNF-α, and NO, along with activities of NOS and caspase-3 in cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of rats supplemented with propolis and subjected to KA mediated excitotoxicity.
Material and Methods

Propolis collection and ethanol extraction

Honey bee propolis was obtained from Muzmi Resources, Kota Bharu, Malaysia. It was subjected to 80% ethanol extract as per the procedure of Isla et al. (2001) as described by Swamy et al. (2014). Single lot of propolis was used for extraction and used for administration to rats.

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 with six rats in each 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

Nitric oxide synthase: NOS activity was estimated by the method of Yui et al. (1991) as described by Swamy et al. (2011a), in which the stable end products, NOx, were estimated using the Nitric Oxide Synthase assay Kit from Calbiochem, U.S.A. (Catalogue Number 482702) The optical density was measured at 540 nm using VersaMax ELISA, Microplate Reader. NOS activity was expressed as nano mole NOx /g wet tissue/ hour

Caspase-3: Caspase-3 activity was assayed using Caspase-3/CPP32 colorimetric assay kit from BioVision Research Products, Milpitas, California, USA. The optical density was measured at 400 nm using VersaMax ELISA, Microplate Reader Caspase activity was expressed as nano mole pNA/g wet tissue/hour

Estimations of NO, and TNF-α: 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). The optical density was measured at 540 nm using VersaMax ELISA, Microplate Reader. TNF-α was estimated using ELISA kit from Abnova GmbH, Germany (Catalog No KA0280). The optical density was measured at 620 nm using VersaMax ELISA, Microplate Reader

Statistical analysis

Results were reported as mean ± standard deviation (SD) from 6 rats for each parameter studied. 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 activity of NOS and NO concentration were increased significantly (p<0.001) in all the three brain regions tested in KA group compared to control group, but the increased activity of NOS and NO concentration by KA were prevented by prior supplementation of propolis (Figure 1 and Figure 2). There were no significant differences in the activity of NOS and NO concentration between control and propolis as well as propolis + KA group (Figure 1 and Figure 2). The concentration of TNF-α 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) (Figure 3). There was no significant difference in TNF-α concentration between control and propolis as well as propolis + KA group (Figure 3).

The activity of caspase-3 was increased significantly (p<0.001) in KA group compared to control and propolis + KA group and the increased activity of caspase by KA was prevented (p<0.001) by supplementation of propolis (propolis + KA group) (figure 4). There were no
significant differences in caspase activity between control and propolis as well as propolis + KA group (Figure 4).

**Figure 1:** The activity of NOS in KA mediated excitotoxicity and propolis supplementation
Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001 versus KA group

**Figure 2:** The Concentration of NOx in KA mediated excitotoxicity and propolis supplementation
Values are mean ± SD from 6 rats
*p<0.001 versus control group; #p<0.001 versus KA group
Discussion

Excitotoxicity is the pathological process where neurons are damaged or killed by overstimulation of glutamate receptors in the Central Nervous System (CNS) by excitatory amino acids and it is considered to be contributing factor in the pathogenesis of various neurodegenerative disorders in the CNS (Wang, et al., 2005; Zhang and Zhu, 2011, Zheng, et al., 2011). It was earlier reported that stimulation of glutamate-KA receptors induces neuronal NO release, which in turn modulates glutamate transmission (Alabadi et al., 1999; Nakaki et al., 2000). NO induces changes in neuronal and signaling-related functions by several ways (Prast and Philippu, 2001). Glutamate excitotoxicity contributes to a variety of disorders in the central nervous system, which is triggered primarily by excessive Ca2+ influx arising from overstimulation of glutamate receptors, followed by disintegration of the endoplasmic reticulum (ER) membrane and ER stress, the generation and detoxification of reactive oxygen species as well as mitochondrial dysfunction, leading to neuronal apoptosis and necrosis (Schinder, et al., 1996; Nicholls, 2004). 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). The production of NO represents one of the principle features of activated macrophage/microglia, and NO is a major effector in the innate immunity (Stuehr, et al., 1991). Earlier work indicated that KA excitotoxicity was associated with energy depletion and oxidative stress (Silva-Adaya, et al., 2008). AMP-activated protein kinase (AMPK), the multifunctional metabolic and energy sensor in the brain, is activated by conditions of cellular energy depletion (Kemp, et al., 1999; Ramamurthy and Ronnett, 2006). It was reported that KA induced activation of AMPK is associated with induction of apoptotic cell death in immortalized mouse hippocampal cells (HT22) and in primary cultures of prenatal rat hippocampal neurons (Ullah, et al., 2011). Korean black bean anthocyanins, naturally occurring antioxidants, were attenuated the neurotoxicity induced by KA in vitro (Ullah, et al., 2011). Their study reported that neuroprotective effects against KA-induced excitotoxicity were attributed to attenuation of numerous KA-induced processes such as ROS accumulation, increase in AMPK activation, perturbation of Ca2+ homeostasis, loss of mitochondrial integrity, inhibition of Bax accumulation, inhibition of the release of mitochondrial cytochrome-c into the cytoplasm, and reduction in cellular content of activated caspase-3 (Ullah, et al., 2011).

Honey bee 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 (Isa 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 (Isa 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 (Isa et al., 2001; Kwon et al., 2004). Propolis contents namely phenolic compounds and CAPE were shown to cross blood brain barrier (Medic-Saric et al., 2009; Silva et al., 2013). CAPE was shown to protects against the loss of dopaminergic neurons induced by 6-OHDA in rats (Silva et al., 2013). Our earlier studies have shown that NOS and NO were increased along with increased oxidative stress markers in KA mediated excitotoxicity and epilepsy rat models (Swamy, et al., 2009, 2011a). We have also reported the amelioration of oxidative stress and NO concentration by propolis supplementation in KA mediated excitotoxicity (Swamy, et al., 2014). Considering that oxidative stress is central to KA induced excitotoxic damage, anti-oxidant and anti-inflammatory treatments may attenuate or prevent the KA mediated neurodegeneration (Zhang and Zhu, 2011). It has been reported that anti-inflammatory substances lucidone (Senthil Kumar et al., 2010), curcumin (Jung et al., 2006), and phenantroindolizidine alkaloids reduce NO production observed in inflammation (Yang et al., 2006). The results of this study showed the prevention of increased NOS activity and NO concentration is in favor of our earlier report (Swamy, et al., 2014). The suppression of proinflammatory marker TNF-α concentration by propolis supplementation in KA mediated excitotoxicity suggests that propolis can protect the neuronal damage by KA. The increased activity of Caspase-3 seen in this study was also prevented by propolis supplementation. Hence the beneficial effects of propolis may be due to its antioxidant, anti-inflammatory and anti-apoptotic properties.

Conclusion

The results of this study showed that propolis supplementation has prevented the increased NOS and caspase-3 activities along with NO and TNF-α concentration in KA mediated excitotoxicity in rat brain. Thus propolis can be a possible potential protective agent against excitotoxicity and neurodegenerative disorders.

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References


