CHAPTER V

DISCUSSION AND CONCLUSION

Estrogen deprivation in menopause could produce learning and memory impairment including increased risk for neurodegenerative disease such as AD (Gao, et al., 1998). Although ERT has a neuroprotective activity and can reduce undesired condition from menopause in women, it has increased risk of serious side-effects such as endometrial cancer, breast cancer and venous thromboembolic events that limit the usefulness of this agent. (Barrett-Connor and Grady, 1998). Therefore, many studies have looked for the new substances which have higher safety and phytoestrogens are attracted interest as a potential alternative to ERT. AR has phytoestrogenic properties and has been thought to be useful for female rejuvenation (Mayo, 1998). The present study, we considered it is interesting to investigate the neuropretective effects of the AR root extract on the animal model of menopause.

Estrogen has neuroprotective effects in several models such as preventing neuronal cell injury, improving performance in memory task of ischemic-induced behavioral deficit in OVX animals (Wappler, et al., 2010; Raval, et al., 2013) and recovering memory deficit by attenuate brain inflammation in rats (Sun, et al., 2013). Earlier document was also revealed that 7 days post-ovariectomy disturbed the performance of object recognition and post ovariectomy for 4 weeks declined spatial memory in rats (Wallace, et al., 2006). The present study found that after ovariectomy for 3 months induced learning and memory impairment indicated by decreasing the recognition index in the NOR test. This finding provides the supporting evidence that ovariectomy affects on recognition memory. Interestingly, our study found that ethanol AR root extract 100 and 1000 mg/kg B.W. as well as EE 0.1 mg/kg B.W. could reverse OVX-induced learning and memory deficit suggesting that AR root extract has neuroprotective properties against recognition memory deficit caused by ovariectomy. Many studies have shown that AR has neuroprotective effects in several animal models. Ojha, et al. (2010) found that AR showed nootropic and anti-amnesic activities via mediating through augmentation of the cholinergic system by anti-

cholinesterase activity (Ojha, et al., 2010). In addition, a rasayana drug containing AR had adaptogenic activity in animals exposed to different kinds of stressors (Rege, et al., 1999).

Loss of memory is associated with neuronal morphological changes have been recognized by several studies. The depletion of neurons and synapses in brain areas mediating memory, such as hippocampus and prefrontal cortex, is characterization of neurodegenerative disease associated with memory deficit, AD. Some studies suggest that estrogen depletion in brain may be a significant risk factor for AD neurophatology (Yue, et al., 2005; Pompili, et al., 2012). Several earlier information have shown that estrogen is a neuromodulator, which mediates the physiological and neuronal function leading to facilitate memory processing through alternations in spine density (Luine and Frankfurt, 2012). The hippocampus and mFPC have been recognized to implicate the recognition memory in the NOR test (Ennaceur and Delacour, 1988). Our results found that 3 months post OVX induced neuronal cells loss in hippocampus (CA1 and CA3 subfields) and mPFC area. These results are consistent with the previous study showing that the Nissl-positive cells staining of three months OVX displayed the reduction of the neuronal density in rats CA3 and dentate gyrus (Takuma, et al., 2007) as same as the results of Su et al. that reported the reduction of neuronal density in CA3 region including loss the volume of hippocampus and neocortex in 4 months post-ovariectomy which were accompanied by spatial memory deficit (Su, et al., 2012). These finding suggested that recognition memory impairment in ovariectomy rats might be associated with neuronal loss in the brain areas of hippocampus and mFPC.

As discussion above, OVX could decline the percentage of recognition index as well as induced neuronal cell loss in the hippocampus and mPFC area. However, administration for 3 months with AR root extracted (100 and 1000 mg/kg B.W.) could reverse OVX-induced morphological loss in hippocampus and mPFC similar to the EE administration. Similar finding was previously observed, an administration of AR root extract (18 ml/kg, 2 weeks) could improve kainic acid-induced neuronal damage and memory loss in mice suggesting that AR enhanced GPx activity and GSH content which resulted in a protective effect (Parihar and Hemnani, 2004). Moreover, neuroprotective effect of the AR methanolic root extract has also been discussed by

Nandagopal et al. and considered that 200 and 400 mg/kg B.W. AR methanolic root extract administration for 7 days rescued the ischemic-induced neuronal damage in rats by its antioxidant activity (Nandagopal, et al., 2011). However, effects of phytoestrogens are not clearly understood but there is several evidences suggest that phytoestrogens could act through estrogen receptors (Kuiper, et al., 1997; Kuiper, et al., 1998; Matthews, et al., 2000). In our study, AR root extract improved memory impairment and prevented neuronal loss induced by ovariectomy as the effect of EE administration. Therefore, it is considered that AR may exert theses effect by its phytoestrogenic activity to be a neuroprotective effect like an exogenous estradiol. These results provide evidence that ability of AR root extract to reduce neuronal morphological changes associating the improving of recognition memory impairment induced by ovariectomy.

It was mentioned above that estrogen available to mediate morphology and physiology of hippocampus and cortex, enhanced cognitive function, and protect neurons from a various kinds of brain insults (McEwen and Alves, 1999; Takuma, et al., 2007; Pompili, et al., 2012). Estrogen has also been recognized for its roles by involvement of BDNF modulating (Solum and Handa, 2002; Scharfman and MacLusky, 2006; Pluchino, et al., 2013). BDNF is widely known to be a member of neurotrophin family, which exerts its effects due to binding with high affinity receptor, tyrosine kinase receptor B (TrkB), and low affinity receptor, p75 neurotrophin receptor (p75NTR). Binding of BDNF to TrkB activates phosphatidylinositol-3-kinase/ protein kinase B (PI3K/Akt) signaling leading to promote neuronal plasticity and neuronal survival essential to process learning and memory (Thoenen, 2000; Yamada, et al., 2005; Driscoll, et al., 2012; Li, et al., 2012).

Based on our results, three months post OVX decreased BDNF protein levels both in hippocampus and frontal cortex. These results were consistent with the previous studies that revealed ovariectomy attenuated BDNF mRNA in the hippocampus (Takuma, et al., 2007) and cortex (Sohrabji, et al., 1995). As previous mentioned above, estrogen has been recognized for its effective in cognitive function via an involvement of BDNF. The evidences suggesting estrogen affects on BDNF was revealed by *in vitro* study that demonstrated 17β-estradiol increased protein levels of BDNF in hippocampal slice cultures (Aguirre and Baudry, 2009). In addition, the

in vivo studies has also been reported that BDNF mRNA levels are significantly reduced in rat hippocampus and cortex after ovariectomy 28 weeks whereas this effect was abolished by estradiol replacement (Singh, et al., 1995). The extensive evidence has shown that estrogen act on ERs to induce BDNF gene transcription. It is understood for mechanism that binding of estrogen on nuclear ERs triggers ERE in the BDNF promoter on DNA to activate gene transcription. Another way, estrogen acts on extranuclear ERs which may trigger signaling pathways leading to phosphorylation of the CREB protein, resulting to BDNF transcription through a cAMP response element in the BDNF promoter (Luine and Frankfurt, 2013). Effects of estrogen on facilitate the neuronal function associating learning and memory improvement was also confirmed by study of Sato and colleagues. They revealed that estradiol increased the postsynaptic density protein-95 (PSD-95) where the PSD-95 is protein associating synaptic formation and plasticity, and also increased the spine density at proximal sites in hippocampal CA3 area. These results have been suggested that estrogen promotes the synaptogenesis by enhancing BDNF synthesis (Sato, et al., 2007). The findings of our study may indicate that estrogen insufficiency produces neuronal morphological lesion associating the reduction of BDNF protein level leading to learning and memory loss.

In addition, we investigated the effects of AR comparative to EE on the expression of BDNF protein in OVX. We found that AR (100 and 1000 mg/kg B.W.) and EE administration for 3 months significantly prevented the following of BDNF protein level induced by ovariectomy in hippocampus while it tended to increase this protein in frontal cortex. However, our results are consistent with the recent studies that the enhancement of the BNDF mRNA level has occurred after administration of soy germ phytoestrogen (1.6 g/kg) for 12 weeks in hippocampus (Pan, et al., 2010) and 150 mg/kg soy phytoestrogens for 8 weeks in frontal cortex in ovariectomy model (Pan, et al., 1999). Previous studied had reported that isoflavones were actives constituent of AR roots (Saxena and Chourasia, 2001). Moreover, the major active constituent of AR root extract is steroid saponins (Shatavarin I-IV) which has phytoestrogenic effects (Bopana and Saxena, 2007). AR root extract in the present study had been evaluated the total saponins by using enzyme-linked immunosorbent assay (ELISA) which revealed the total saponin was 7.42%. Other study has reported

that ginsenoside Rg1, a steroidal saponin of high abundance in ginseng, were also known for its phytoestrogenic effects (Chan, et al., 2002; Lee, et al., 2003) and it could reverse the corticosterone-induced changes in mRNA levels of BDNF in hippocampal mice (Chen, et al., 2014). In the present study, AR root extract and EE increased BDNF protein levels in the hippocampus and frontal cortex of OVX rats suggesting that AR might exert these effects by depending on phytoestrogenic activity to increase BDNF protein expression. These effects leaded to prevent the morphological changes associating attenuation learning and memory impairment induced by ovariectomy.

Several investigations have extensively considered that fluctuation of circulating estrogen levels have an affect on learning and memory ability. They found that enhancing memory was occurred during highest levels of estrogen in proestrus in rodent and fluctuated across menstrual cycle correlating positively with serum estrogen levels (Frick and Berger-Sweeney, 2001; Rosenberg and Park, 2002; Korol, et al., 2004; Spencer, et al., 2008). Although AR root extract had shown neuroprotective effects similar to exogenous estradiol, it had no effect on serum estradiol concentration in OVX in the present study. Similar to previous study, Menosan, a herbal preparation containing AR and used to improve symptoms of menopause, had uterotrophic activity by increasing the uterine weight without an effect on serum estradiol in OVX rats (Gopumadhavan, et al., 2005). Moreover, our findings also reliable with the previous studies that investigated the effects of U-3107 (the herbal formulation containing AR 6.4%) on uterine weight and serum estradiol level. They found that U-3107 (1 mg/kg) administration for 21 days increased uterine weight in normal rats but not in OVX animals. It has been considered that U-3107 possesses uterotrophic activity only in the presence of a functional ovary (Mitra, 1999).

The present study did not find AR affected on serum estradiol concentration in the OVX but AR could improve recognition memory associating the changes of BDNF protein and neuronal morphology. These results were considered that AR may process its effect to prevent the memory loss in estrogen insufficiency due to another mechanism. Estrogen processes its neuroprotective effect by binding with classical ER subtypes, ERα and ERβ. These receptors are presented in the hippocampus and frontal cortex where these regions specifically associated with recognition memory

(Shughrue, et al., 1997; Towart, et al., 2003). Previous investigation showed that BDNF and TrkB including PSD-95 mRNA were decreased in ERα and ERβ knockout mice (Spencer-Segal, et al., 2012). Therefore, it was considered that both of ERα and ERβ played an important role on hippocampus functions.

The present study demonstrated that down-regulated ERs protein expression in both the hippocampus and frontal cortex was found after 90 days of OVX. We have been obtained several evidences indicating that estrogen affects on the expression of ER subtypes. One of them is the investigation of Romeo and co-workers that showed ERα in the hippocampal dendritic spines in proestrus is higher than in diestrus phase of rats (Romeo, et al., 2005). In addition, the study in the changes of ERs expression in rat brain has considered that ERβ in cortex significantly declined with aging (Wilson, et al., 2002). The mechanisms of estrogen to mediate their receptor status remain unclear. However, there was a suggesting one conserved function of steroid hormone receptors are autoregulation of their own gene expression (Schmidt and Meyer, 1994).

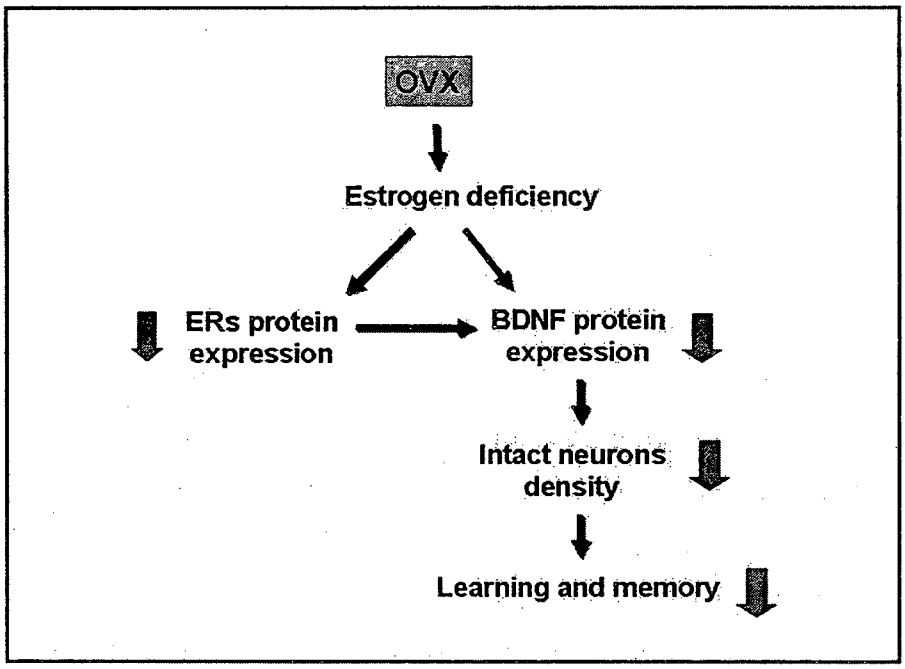
Our result of down-regulation of ERs proteins in OVX was not consistent with the study of Cardoso and colleagues that OVX 15 days produced ERα up-regulation in the rat hippocampus (Cardoso, et al., 2010). However, our findings in accordance with the recently study that demonstrated a decrement of ERα in rat cortex and hippocampus after long-term of ovariectomy (6-24 months) (Navarro, et al., 2012) and an amelioration of ERβ in the brain of 3 months OVX rats (Rose'Meyer, et al., 2003). These results may be considered that a short-term of ovariectomy causes a short period for a lack of circulating estrogen which results in a compensatory up-regulation of ERs for physiological responses. On the other hand, the lack of estrogen for 3 months has supported the idea that long-term of estrogen insufficiency leads to down-regulation their receptors in the brain. Accordingly, Navarro et al also reported that E2 administration abolished OVX-induced ERα decline similar to our findings which EE treatment inhibited the decreasing of ERs. It is reasonable to support that estrogen play an important role against ERs down-regulation in brain.

In the present study, ethanol AR root extract (1000 mg/kg B.W.) could prevent the decreasing of ERα protein in hippocampus and frontal cortex as the effect of EE. Furthermore, it was also revealed that ethanol AR root extract (100 and 1000 mg/kg B.W.) restored a down-regulation of ERβ in OVX as same as EE in both hippocampus

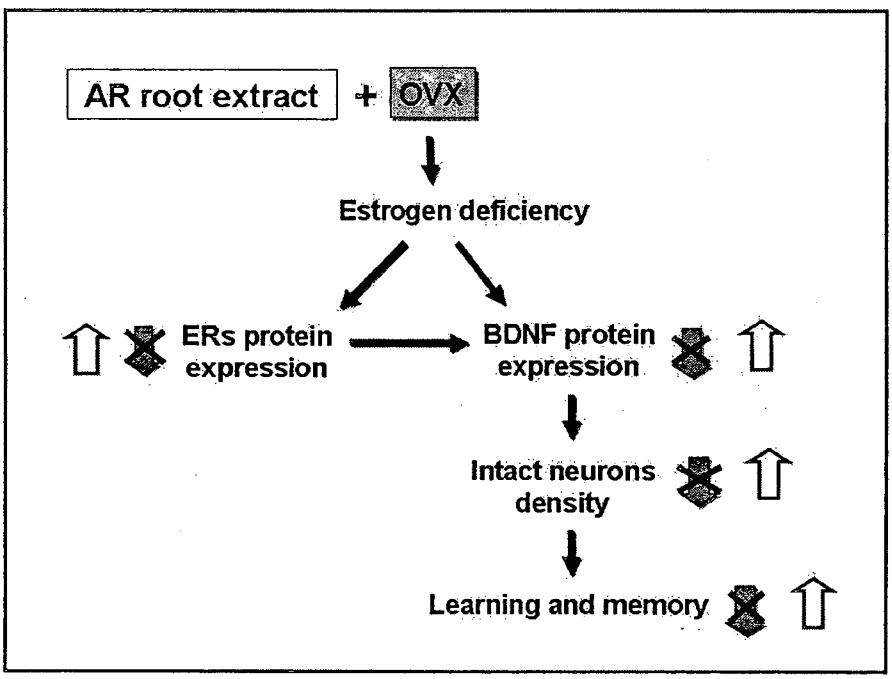
and frontal cortex. Phytoestrogenic properties of AR are wildly known and used as a hormonal modulator in a herbal stimulant health tonic for women (Saxena, et al., 2010). Although, phytoestrogenic effects are poorly understood, it could act through estrogen receptors (Kuiper, et al., 1997; Kuiper, et al., 1998; Matthews, et al., 2000). Previous study has reported that coumestrol, one class of phytoestrogens, could mediate the expression of ERβ in the paraventicular nuclease of hypothalamus (Patisaul, et al., 1999). In addition, previous study exhibited that dietary intake of phytoestrogens up-regulated ERα in breast tumor cell in premenopausal women (Touillaud, et al., 2005). As our results, ethanol AR root extract protected down-regulation of ERs. It was explained that AR may mediate its activities through ERs binding and trigger the ERs expression to process the protective effects in the OVX.

Summary and conclusion

As the results, the present study demonstrated that OVX produced learning and memory impairment associated neuronal cell loss. These changes related to ameliorate of estradiol levels including the expression of BDNF and ERs proteins. These results in the present study were considered that OVX induced the downregulation of ERs proteins which resulted in reduction of BDNF protein expression. This decrement might cause neuronal injury and death in the hippocampus and mPFC where associated with recognition process (Figure 23 (A)). However, the administration of the ethanol AR root extract could obviate the OVX-induced learning and memory loss as well as neuronal damage in hippocampus and mPFC. Although, AR had no effect on estradiol levels, it was found to ameliorate the reduction of ERs and BDNF protein expression induced by OVX. Therefore, these results suggest that protection of ethanol AR root extract against OVX-induced learning and memory loss was accounted by its ability to increase the expression of ERs and BDNF proteins which were diminished by OVX (Figure 23 (B)). However, further investigations are required to understand the possible involvement of another mechanism for improving property of AR.



(A)



(B)

Figure 23 The mechanism of OVX on learning and memory (A) and the protective effect of ethanol AR root extract against learning and memory impairment (B)