CHAPTER V
DISCUSSION

5.1 Short-term effects of estradiol and CHE on the spatial reference memory of ovariectomized rats

Ovariectomized rats were used as the animal model to mimic the hormone deficiency situation of menopause and its effect on the spatial memory. A pre-OVX MWM training was given to the rat. The present study demonstrated that OVX group tend to have the impaired spatial memory but not statistically different when comparison of average acquisition time. However, not as expected, estrogen replacement did not facilitate the effect due to the OVX, nor did the CHE. Both estrogen and CHE treatment augment the OVX effect, making the OVX rats have the higher acquisition time which indicates an impaired memory. One of the reasons might support these results is the dose of the treatment. The effect of estrogen on the memory related behaviors test of the rodent is dose various. Low concentration of estrogen administered (i.e. 0.3 μg) alleviated the impaired memory due to the OVX but the high dose of the estrogen (i.e. 5-10 μg) impaired the memory (Holmes, Wide & Galea, 2002; Wide, Hanratty, Ting & Galea, 2004) . In the present study, estrogen at the dose of 10 μg/kg of the body weight (around 2 μg/dose) was not as high as the dose criterion in the previous reports, but the daily administered estrogen right after the OVX might cooperate with the storage-inside-body endogenous estrogen and consequently result in a higher estrogen concentration than the physiology level. The post-OVX blood estradiol level in rats was reported to maintain at 30% percent of its normal level at the 11^{th} day and around 20% of its normal level at the 4^{th} weeks after the OVX (Chu *et al.*, 1999). The estrogen was reported to be generated from the nongondolas subcutaneous abdominal adipose tissues, liver tissues and the brain.

5.2 RT-PCR quantification of the estrogen receptor mRNA in the hippocampus of the rats.

The mechanism of how estrogen affecting the ER mRNA is not clear, but the consequence of the effect of exogenous estrogen, includes phytoestrogen, had been

reported (Gundlah *et al.*, 2000; Jin *et al.*, 2005). The mRNA stability contributes to the ER expression in different tissues, and the ER had been proved to act as an essential role in the ovarian hormone signal transfer to the nuclear of target tissues. The present study showed the increasing effect of estradiol to both ER alpha and beta mRNA level in OVX rat hippocampus, which is different from the other studies (Saceda *et al.*, 1998; Suzuki & Handa, 2004) . This discrepancy may be due to the result of different dose, administrated route and the period of treatment. On the other side, the increasing effect proved the up-regulate activity of estrogen to the ER mRNA. The CHE administration also up-regulated the ER alpha, but not beta level in the OVX rat hippocampus, indicating the CHE had the estrogenic like function in the brain with the selectivity to the ER alpha. We also notice that the effect of CHE on ER alpha mRNA level exist only on the OVX rats, not on normal rats. This phenomenon may reflect the different binding ability of estradiol and CHE to the ER, when the estrogen is exists, it dominates the ER regulation compare to our candidate phytoestrogen. This result is concordant to the combine ability test of which had proved that estradiol had higher combine ability to ER than CHE (Piyachaturawat *et al.*, 1995a). The selectivity effect showed the dose dependence, even when the mRNA level is two times higher than the levels of normal rat. This much-higher-than-normal level increase may be due to the lack of the related metabolite or down regulation mechanism similar to the estrogen which had been develop in the body. This should raise the consideration of the dose in the clinical trial, especially with the estrogen deficiency patients.

5.3 Long-term effect of estradiol and CHE on the spatial reference memory and working memory of ovariectomized rats

CHE increasing the MWM test performance at dose dependence for the memory impaired rats due to OVX was consistent with the hypothesis of the present study. The estrogenic-like function of CHE was confirmed by the increased uterus weight of OVX rats at postmortem, which also showed a dose dependent manner. The OVX rats that received a high dose CHE performed the best, even better than the ovary intact rat, for both the hidden platform test and the probe test. These cognitive enhancing effects

were evident at the 1st period. At the 3rd and 4th day of the learning process, the OVX+C2 groups showed a shorter latency and distance to the platform, which was close to the level the other groups achieved at the 2nd and 3rd period. The CHE administration also increased the active time of the animals at the MWM performance, especially at the probe test when compared to the other groups, which indicated a higher motivation to find the platform. This evidence provided strong support for many pre-published papers by which the effects or the potential effects of phytoestrogen on the cognition improvement were mentioned (Belcher & Zsarnovszky, 2001; Lephart, Setchell & Lund, 2005; Luine *et al.*, 2006).

Another objective in the present study was to clarify whether the OVX would affect the spatial reference memory and spatial working memory, and when these effects would happen after the OVX in the female rats. The result of OVX impaired the spatial reference memory on the female rat were consistent with most of the previous reports (Daniel & Lee, 2004; Daniel, Roberts & Dohanich, 1999; Packard & Teather, 1997). The memory impairment of the OVX rats was evident at 67 days after the surgery, during the hidden platform test of the 2nd period of MWM test (M-H2). However at the M-H1 (30 days after the OVX), no significant difference was found between OVX and the other groups. Of special note, the M-P1 test, which was performed 2 days earlier than the M-H2, did not show a difference between OVX+V and the other groups that occurred at the adjacent M-H2 test. These results could indicate that the memory degeneration on the OVX rat at 67 days was related to the daily learning process but was not a forgotten platform location learned at 30 days ago. At the 2nd and 3rd probe test (M-P2 and M-P3, 92 days and 119 days after OVX), the scores of the OVX+V group were lower than the other groups, compared to M-H2 and M-H3, but it was difficult to say whether these lower scores were due to the lower learning ability during training or due to long term memory loss when compared to the 1st period. Through longitudinal analyses, the progress of each group from the 1st period to the 3rd was compared to evaluate the memory or learning condition. The absolute score recorded from the performance was not used, since the animals were also learning the rules of the game and adapting to the environments concurrent to the testing. This could have resulted in achieving better scores at the later test. This

evidence could offer insight into the oncology of neuron degeneration in menopausal or post menopausal conditions with hormone deficiencies.

Young, middle aged rats (6-10 months) were chosen because the current objective was to investigate the effect of estradiol on the memory of a fully developed brain, and the factor of age would not complicate the analyses. At this age, the hormone level in the rats is stable. Rats older than 10 months would begin to have fluctuations in estrogen levels and then gradually decrease. In animal centers, female rats used for production of offspring normally discard females at the age of 10-11 months. OVX of the 6 month old, adult rat also matched our objectives of investigating the effects on the fully developed brain but not on the developing one. The dose of estradiol and the route of the administration were referenced from the previous reports (Butcher, Collins & Fugo, 1974; Garza-Meilandt, Cantu & Claiborne, 2006; Geary & Asarian, 1999; Henderson, Baker, & Fink, 1977a, 1977b; Hu & Becker, 2008) . The dose of 2 μg/day subcutaneous injection was designed to mimic the physiological estrogen level. The estrogenic effect was proved in a previous study and the present study, where the uterine weight of the OVX rats significantly increased under this dose and administration route. As expected, the estradiol treatment attenuated the impairment induced by the OVX which began at the 67 days, helping the animal to achieve a higher score in the MWM test.

The swimming speed of each group was consistent across the 3 periods, indicating the OVX and the designed treatment did not affect the motor ability of the animal, which might have affected the performance of a motor-dependent behavior test. The changes in the active time might be due to the motivation lost or the memory of the platform location lost.

The performance of ovary intact rats at the M-H2 was considerable, since this group did not progress in their performance across the 5 learning days. Although they had a better performance at the 1st and 2nd day compared to the OVX+V group. Food deprivation might have caused this effect. Only the M-H2 was designed to follow the food deprivation, which was from the RAM1 when compared to the M-H1 and M-H3. These results could be due to the estrus cyclicity that was disrupted by the food deprivation, which ultimately affected the memory (Daniel *et al.*, 1999) .

The OVX did not show significant effects on the RAM test, for either the 1st and 2nd period. The OVX+ vehicle group made the fewer errors at both the free accessible test and delay non-match to sample test at 10 minutes, 1 hour and 2 hours delay. This result was similar to that reported by (Ziegler & Gallagher, 2005) but inconsistent with some of the previous reports (Daniel, Hulst & Berbling, 2006; Luine *et al.*, 1998). The difference could be from the different experiment design, which probably related to the age of the animal. The young OVX rats (25 days old) (Daniel *et al.*, 1999) and old OVX rats (17 month) (Daniel *et al.*, 2006) both showed an enhanced effect on the RAM performance by the estradiol replacement when compared to the non-treated group. These results indicated that the estrogen deprivation due to the OVX itself would not affect the working memory of a matured rat brain, but had the effect on the developing brain or an ageing brain which that be attenuated by the estrogen replacement.

The effects of CHE on the RAM performance were reverse dose dependent. The OVX+ low dose CHE group made more errors, while the delay time increased at the DNMTS test, but the OVX+ high dose extract animal outperformed other groups. This phenomenon is difficult to explain under the current experimental design.

5.4 Brain morphology study

Morphometry had been used for evaluating the brain morphology changes related to the age and pathology in human. Positron emission tomography (PET) and magnetic resonance imaging (MRI) techniques are used for obtaining the 2-dimention images for the following volume based morphometry. However, this technique has the limitation on the small-size rat or mice brains. PET imaging reflects function but not primarily structure, and therefore, reveals few landmarks in the brain or skull. MRI imaging shows a variable degree of structural detail, depending on the specifications of the instrument and the imaging protocols so that a common challenge is therefore to assign locations to the data collected with use of available brain atlases mostly identified by AChE and Nissl staining. In present experiment, the technique of section-staining-reconstruction allows us to segment much more and detail areas we are interesting in according to the widely used rat brain atlas (Paxinos

& Watson, 2004) and then reconstruct them and show the volume. In our knowledge it's the first time to reconstruct the rat brain from the AChE staining section and provide the volume of the structures. The accuracy of the volume quantification could be proved from the data of the absolute hippocampal volumes, which were related to the bodyweight in both data of groups and single animal ($P<0.1$) (Figure 97-98).

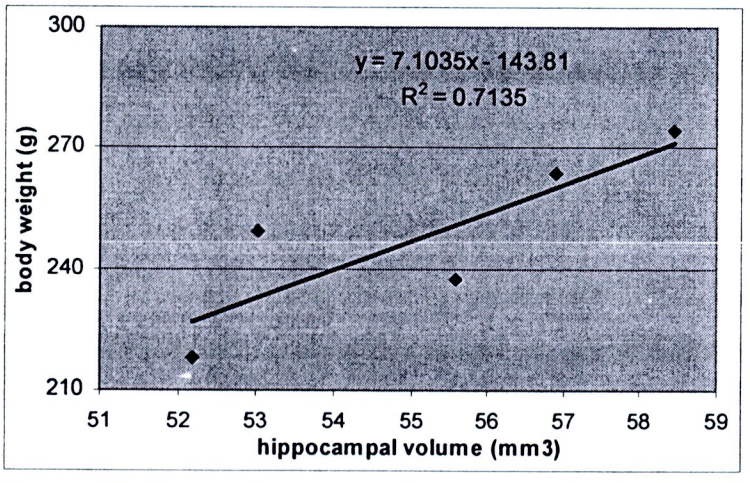

Figure 97 Regression curve of hippocampal volume and body weight from 5 groups of animals

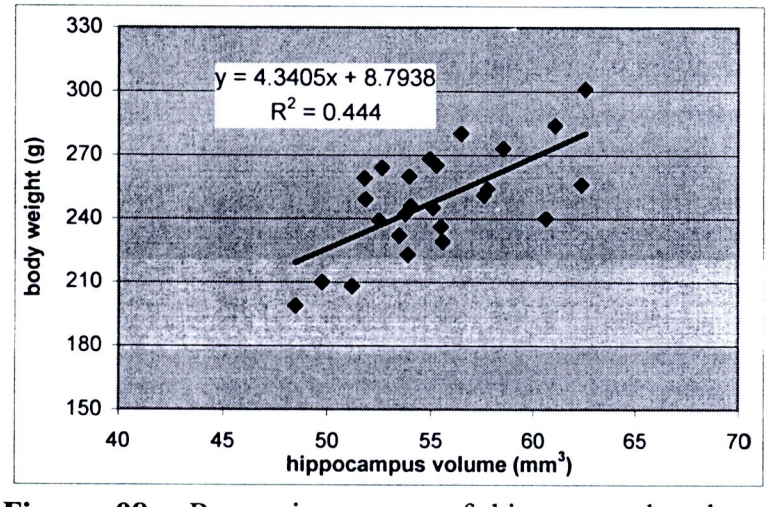

Figure 98 Regression curve of hippocampal volume and body weight from individual animal.

The absolute hippocampal volume of each group did not match to the memory test results, for example, the groups having good memory should have the bigger

hippocampal volume, according to the brain volume also related to the body weight. After compensating by the body weight, the proportion of the hippocampal volume between groups is quite comparable to the results in the long term spatial reference memory study, which indicates the impaired memory due to the OVX is related to the morphology integrity of the hippocampus, the priority structure respond to the spatial memory in the brain. The administration of estrodiol and CHE can reverse this effect.

Similar to the hippocampal volumes, the neuron number in the CA1 area is related to the body weight and in wherever the anterior, middle or posterior of hippocampus. The CHE and estradiol treatment affect the CA3 area more than the CA1. The absolute number of the neuron in the CA3 area of OVX+C2 and OVX+E groups are closed to the control group, even they have the lowest body weight. Meanwhile, the OVX group had the lowest absolute neuron number in CA3 although they are the heaviest. These differences are amplified after compensating by the body weight result in the highest CA3 neuron number in the OVX+C2 group followed by the OVX+E, which also support the behaviors results. The more effects in CA3 than CA1 neuron numbers indicate the estrogen and CHE could improve the signal encoding process of the spatial memory in the OVX rat

However, the limitation of using the relative hippocampal volume and neuron numbers should be aware since the proportion of body weight loss due to the treatment is not clear yet in the present study. Especially in the CHE treated group; the body weight loss might be due to the effect from some other compounds in the crude extract through a non-estrogenic pathway in addition to the estrogenic pathway. These may involve the lipid or sugar metabolism, which would result in higher relative hippocampal volume and the neuron numbers.

5.5 The effect of the CHE on the antioxidative enzyme activities in the rat brain against the ethanol-induced oxidative stress

The effects of ethanol as an oxidative stress inducer on the antioxidant enzymes in the rat brain were observed in different tissues. The significant increase of SOD activity in the cortex and GPx activity in hippocampus and hypothalamus are consistent to the previous report (Somani *et al.*, 1996) . However, reversed result was

observed for CAT activity, i.e. decreased in the cortex but increased in the hypothalamus. The differences might be due to the duration of the administration. In the paper of Somani *et al* (1996), sampling was 15 minutes right after the ethanol administration; whereas 2 weeks treated time was designed in the present study, indicating the different influence on the CAT activity of acute and chronic effect. For the chronic ethanol intoxication effect, ethanol was report to decrease the total antioxidant ability in the brain of the rats after 2 months consumption (Kurban, 2008).

Various activities in different tissues of each enzyme indicated the variety of the ROS generated by ethanol in these tissues. The higher activity of SOD in the cortex indicated the abnormal $O_2^{\cdot-}$ in this tissue, where the CAT and GPx had the low activity. In the hypothalamus and hippocampus, higher activities of CAT and GPx were observed while the SOD activity was low, indicating the oxidative stress was from H_2O_2 but not $O_2^{\cdot-}$. These could be related to the metabolic process of ethanol in the brain, which was not totally clear today. These anti-ethanol-induce-oxidative abilities are believed to be helpful in increasing the defend system to the oxidative stress in the brain. However, it is hard to relate to the previous behaviors results in the present study.

The hypothetical anti-oxidative effect of CHE to the ethanol was observed but not in all the time. For example, the effects on the GPx in hypothalamus and hippocampus were obvious but not in pituitary and cortex. The augment effect to the ethanol was happened as in all the test tissue, SOD activity was higher in the ethanol and CHE co-treated groups than the ethanol only. These anti-ethanol and augment effect were complicated and may be due to two reasons in brief. The first one is the CHE involved in the ethanol oxidative process in the brain, for example indirectly increase the substrates in the reaction such as NADPH or H_2O_2, which would help to increase the enzyme activities. Another possibility is the natural anti-oxidative CHE could react with the ROS generated from the ethanol metabolism in the brain and keep the antioxidant enzyme not too busy. It seems that the first reason is more prefer, since the CHE did not change the enzyme activity at most of the time in the case of no ethanol was treated.

5.6 Pharmcokinetics study of the CHE in the rats

The pharmacology effects of the CHE has been previously reported in the published paper and shown in the present study. To conduct a more delicate experiment, the more purified active compounds from this crude extract are required, which is also the critical step for developing this effective plant extract to be a potential candidate for the clinical study. The traditional way, separate and purify the compounds by the chromatography method and then screen the active one from them, identifies the active compound by excluding the non-effectives, which is invalid and not necessary. In the present study, the crude extract was administered to the animal to identify the compounds can be absorbed from the GI track, followed by measuring their pharmacokinetic parameter such as the distribution in the brain or reproductive systems to evaluate their potentials to be an active one for the further study. This *in vivo* exposure screening method excludes several non-absorbed compounds, making the active compounds screening process more efficiency.

To select the dose for this study, both the sensitivity of the HPLC detector and the clearance time by the GI track were considered. Too low dose was resulted in the increase of the undetected time points, which would affect the analysis precision of the compounds with minor concentrations. The higher dose made the longer absorbed time so that the analysis time point had to be increased. At the present dose design, the CHE could be cleared at around 12-16 hours after the oral feeding by observing the color in the GI track and smelling its unique odors at the autopsy.