ผลของยาต้านตัวรับแอดรีเนอร์จิกชนิดเบตา (คาร์วีไดลอล และ โพรพราโนลอล) ต่อตัวรับแอดรีเนอร์จิกชนิดเบตา 2 และระดับของแฟกเตอร์ VIII ในแมวที่มีระดับไทรอยด์ฮอร์โมนในเลือดสูง

นางสาว จันเพ็ญ บางสำรวจ

สถาบันวิทยบริการ
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2549

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF BETA-ADRENERGIC ANTAGONISTS (CARVEDILOL AND PROPRANOLOL) ON BETA 2 – ADRENERGIC RECEPTORS AND FACTOR VIII LEVELS IN HYPERTHYROID CATS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Effects of beta-adrenergic antagonists (Carvedilol and Propranolol) on beta 2 adrenergic receptors and factor VIII levels in hyperthyroid cats.
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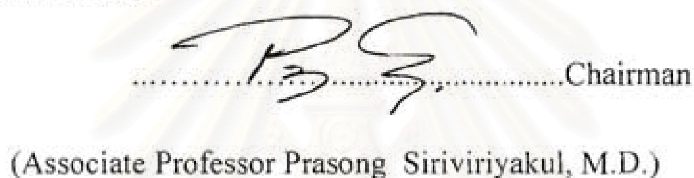
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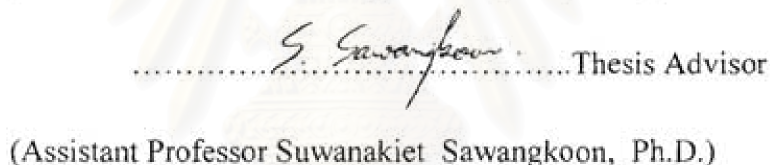
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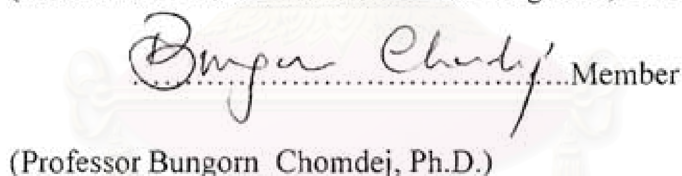
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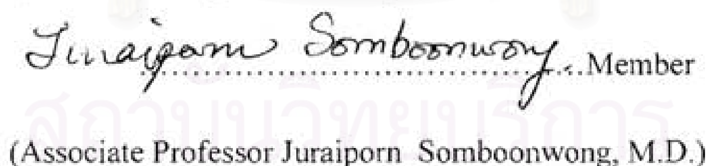
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จันเพ็ญ บางสำรวจ : ผลของยาต้านตัวรับแอดรีเนอร์จิกชนิดเบตา (คาร์วิไดลอล และ โพรพราโนลอล) ต่อตัวรับแอดรีเนอร์จิกชนิดเบตา 2 และระดับของแฟกเตอร์ VIII ในแมวที่มีระดับไทรอยด์ฮอร์โมนในเลือดสูง (EFFECTS OF BETA-ADRENERGIC ANTAGONIST (CARVEDILOL AND PROPRANOLOL) ON BETA 2 – ADRENERGIC RECEPTORS AND FACTOR VIII LEVELS IN HYPERTHYROID CATS) อ. ที่ปรึกษา : ผศ.นสพ.ดร.สุวรรณเกียรติ สว่างคุณ, 62 หน้า

การวิจัยนี้มีจุดมุ่งหมายเพื่อเปรียบเทียบผลของยาต้านตัวรับแอดรีเนอร์จิกชนิดเบตาต่อระดับแฟกเตอร์ 8 และตัวรับแอดรีเนอร์จิกชนิดเบตา 2 ในแมวปกติและแมวที่มีระดับไทรอยด์ฮอร์โมนในเลือดสูง การวิจัยนี้ใช้สัตว์ทดลองจำนวน 12 ตัว ประกอบด้วย กลุ่มควบคุมจำนวน 4 ตัว และกลุ่มที่เหนี่ยวนำให้มีระดับไทรอยด์ฮอร์โมนในเลือดสูงจำนวน 8 ตัว กลุ่มที่มีระดับไทรอยด์ฮอร์โมนในเลือดสูงนั้นจะได้รับแอลไทร็อกซีนในขนาด 50 ไมโครกรัม/กิโลกรัม/วัน เป็นเวลา 14 วัน หลังจากนั้นในแต่ละกลุ่มจะถูกสุ่มเลือกมาเพื่อให้ยาโพรพราโนลอลหรือ คาร์วิไดลอลก่อน โดยครึ่งหนึ่งจะได้รับยาโพรพราโนลอลในขนาด 1 มิลลิกรัม/กิโลกรัม/วันร่วมกับแอลไทร็อกซีน เป็นเวลา 14 วัน แล้วเข้าสู่ระยะพัก 14 วัน ต่อจากนั้นจึงให้คาร์วิไดลอลในขนาด 1 มิลลิกรัม/กิโลกรัม/วันร่วมกับแอลไทร็อกซีน เป็นเวลา 14 วัน ส่วนที่เหลือก็จะได้รับคาร์วิไดลอลก่อนแล้วตามด้วยโพรพราโนลอล หลังจากนั้นทำการเก็บตัวอย่างเลือดเพื่อนำไปวัดระดับไทร็อกซีน, แฟกเตอร์ 8, ค่า PT, APTT และจำนวนตัวรับแอดรีเนอร์จิกชนิดเบตา 2 ในเม็ดเลือดขาว ผลการศึกษาพบว่า ในกลุ่มที่มีระดับไทรอยด์ฮอร์โมนในเลือดสูงมีระดับของแฟกเตอร์ 8 สูงร่วมด้วย คาร์วิไดลอลสามารถลดระดับไทร็อกซีนและแฟกเตอร์ 8 แต่โพรพราโนลอลสามารถลดระดับไทร็อกซีนได้เพียงอย่างเดียว แต่ไม่มีการเปลี่ยนแปลงจำนวนของตัวรับแอดรีเนอร์จิกชนิดเบตา 2 ภายหลังได้รับไทร็อกซีน คาร์วิไดลอล หรือ โพรพราโนลอล ผลการทดลองชี้ให้เห็นว่า แฟกเตอร์ 8 ซึ่งสัมพันธ์กับการเกิดภาวะลิ่มเลือดอุดตันสามารถลดระดับลงได้โดยการให้ยาคาร์วิไดลอล

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สาขาวิชา........สรีรวิทยา (สหสาขาวิชา)............ลายมือชื่อนิสิต..

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KEY WORD : CATS/HYPERTHYROID/FACTOR VIII/BETA 2 ADRENERGIC RECEPTOR/CARVEDILOL/PROPRANOLOL/ADRENERGIC ANTAGONISTS.

JANPEN BANGSUMRUAJ : EFFECTS OF BETA - ADRENERGIC ANTAGONISTS (CARVEDILOL AND PROPRANOLOL) ON BETA2–ADRENERGIC RECEPTORS AND FACTOR VIII LEVELS IN HYPERTHYROID CATS. THESIS ADVISOR : ASSISTANT PROFESSOR SUWANAKIET SAWANGKOON, Ph.D., 62 pp.

The objective of this study was to compare effects of β_2 adrenergic blockers (carvedilol and propranolol) on factor VIII levels and β_2 adrenergic receptors in normal and hyperthyroid cats. Twelve cats were divided into two groups, a control group (n= 4) and a hyperthyroid group (n= 8), which animals in the hyperthyroid group were given L-thyroxine at the dose of 50 μg/kg/day for 14 days. After an induction period, all animals were randomly selected to treat first with either propranolol or carvedilol. Half of animals received orally 1 mg/kg/day of propranolol combination with L-thyroxine once daily for a total period of 14 days. After that, animals were allowed to rest and washout for 14 days, and then they were received L-thyroxine for 14 days followed by L-thyroxine combination with 1 mg/kg/day carvedilol once daily for 14 days. Another half received carvedilol; then followed by propranolol. Blood samples were drawn for determinations of thyroxine levels, factor VIII levels, prothrombin time (PT), activated partial thromboplastin time (APTT), and β_2 adrenergic receptor density. It was found that the hyperthyroid animals shown an increase in factor VIII levels. Carvedilol decreased thyroxine and factor VIII levels, but propranolol decreased only thyroxine levels. There was no change on the density of β_2 adrenergic receptors after thyroxine induction and after treated with either carvedilol or propranolol. These results indicate that factor VIII, which is related to thromboembolism, can be decreased by carvedilol.

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Field of study...Physiology (Inter-Disciplinary Program)…Student's signature..........

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LIST OF ABBREVIATIONS

APTT	Activated partial thromboplastin time
B2 ADR	beta 2 adrenergic receptor
BW	body weight
Ca^{2+}	Calcium ion
CBZ	Carbimazole
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
Kg	kilogram
KH_2PO_4	Dipotassium hydrogen phosphate
µg	microgram
mg	milligram
ml	mililiter
mM	milimolar
MMI	methimazole
Na^{+}	Sodium ion
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
PBS	Phosphate buffer saline
PT	Prothrombin time
PTU	Propylthiouracil
sec	second
TBG	thyroxine binding globulin
TBPA	thyroxine binding prealbumin
t-PA	tissue plasminogen activate

TRE	thyroid receptor element
TTR	transthyretin
vWF	von Willebrand factor

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CHAPTER I

INTRODUCTION

Functions of thyroid hormones produced by thyroid glands are to regulate the basal metabolism of all cells, and synthesize proteins, carbohydrates and lipids which are important for the normal organ growth, development and function (Tortora and Derrickson, 2006). Thyroid hormones produce nuclear transcriptions of large numbers of genes, enzymes, structural proteins, and transport proteins, which result in a generalized increase in functional activity throughout the body (Guyton and Hall, 1996).

An increase in protein synthesis of all cells resulting from thyroid hormone activities contributes to an increase in the sensitivity of adrenergic receptors on catecholamine response (Bilezikian and Loeb, 1983). This phenomenon has been explained that thyroid hormones can modulated the number of adrenergic receptors by enhance protein synthesis (Tse *et al*., 1980), and many studies have found that triiodothyronine induces an increase in the mononuclear leukocyte β adrenergic receptor density in humans (Ginsberg *et al*., 1981). On the contrary, thyroidectomized patients have a decrease in β adrenergic receptor numbers when compared to hyperthyroid patients (Cognini *et al*., 1983). This change has indicated that an enhancement of adrenergic receptors can alter the coagulation system by increasing factor VIII levels in blood (Gardikas *et al*.,1968 and Ingram *et al*., 1977), suggested from a β_2 – adrenergic effect (Ingram *et al*., 1977). This effect was later confirmed by several studies, such as Von Känel et *al.* (2002a) found that adrenergic stimulation of the vascular endothelial β_2 receptor caused an increase in clotting factor VIII activities, by releasing molecules from endothelial storage sites into the circulation. Therefore, hyperthyroidism may cause an increase in the incidence of thrombosis in human subjects (Horne *et al*., 2004). The most commonly reported thromboembolic events are emboli related to atrial fribrillation (Presti and Hart, 1989). Mechanisms responsible for this are including the high level of factor VIII (FVIII) during the hyperthyroid period (Rogers and Shane, 1982).

There is a study which has shown that non-selective beta blockers (β_1, β_2) such as propranolol can decrease factor VIII activities (Roger and Shane, 1983). Recently, there is a newer generation of beta blockers, which drugs effect on hemostasis is still not known, e.g. carvedilol, which possesses β_1, β_2 and α_1 adrenergic blockades without up regulation of β receptors (Gilbert *et al.*, 1993). This drug is introduced to the market and may be used substitution of the older generation. Yet, this needs to be confirmed for their hemostatic actions before used.

Hyperthyroidism causes an increase in coagulation factor activities, contributed to an increase in a risk factor for thrombosis. This disorder is associated with an incidence of 8-40% of arterial embolism, especially cerebral thromboembolism in humans (Erem *et al.,* 2002). The high levels of factor VIII in the circulation are associated with an increase in the risk of thrombosis (Kyrle *et al.,* 2000). Moreover, the factor VIII activities in cats were reported 13 fold higher than in humans (Mischke *et al*., 1995), which may be associated to the development of hemostatic complications, e.g. feline distal aortic thromboembolism. This complication is one of the serious complications associated with feline cardiomyopathy that produces hindlimb paresis and live threatening (Flanders, 1986). Thus, drugs that possess adrenergic blockade leading to a decrease in factor VIII activities may decrease hemostatic complications and prolonged life (Kyrle *et al*., 2000).

The use of beta blockers may be one of several choices for prevention and treatment of hypercoagulation state in hyperthyroid patients as well as decreased incidence of thrombosis. Despite all these facts, this issue has never been investigated in cats. We hypothesize that hyperthyroidism may increase beta adrenergic receptor density and factor VIII, and these changes may be declined by carvedilol and propranolol in different degrees.

Therefore, the present study was designed to:

1. Examine the effects of thyroid hormones on the beta 2 adrenergic receptor densities of mononuclear leukocytes and factor VIII levels in cats.

2. Compare the effects of carvedilol and propranolol on the beta 2 adrenergic receptor densities of mononuclear leukocytes and factor VIII levels in cat

CHAPTER II

LITERATURE REVIEWS

This section provides the background information for the study being reported. It serves the following information:

A. Thyroid hormones
   - Hyperthyroidism in cats

B. Adrenergic receptors

C. β adrenergic antagonists

D. Coagulation system

E. Coagulation disorders in thyroid diseases
   - Relation of hyperthyroidism with factor VIII levels and thrombosis.

F. Effect of beta blockers on thrombosis

A. Thyroid hormones

Thyrotropin releasing hormone (TRH) is a regulator for thyroid stimulating hormone (TSH) secreted by hypothalamus. It governs amount of TSH produced from the anterior pituitary gland which plays role on thyroid hormone productions e.g. thyroxine and triiodothyronine. The levels of both thyroid hormones may be controlled by the way of negative feedback inhibition of the synthesis of thyrotropin releasing hormone at the level of hypothalamus and followed by inhibition of TSH secretion at the pituitary gland as shown in figure 2.1.

In the thyroid glands, follicular cells produce thyroid hormones which comprise of triiodothyronine (T_3) and tetraiodothyronine (T_4). Both hormones have two major physiological effects which regulate metabolism and increase protein synthesis (Berne *et al*., 2004).

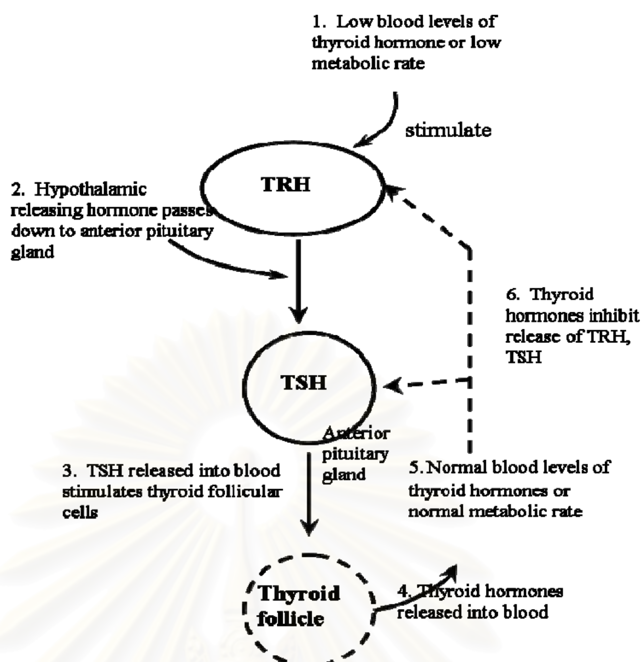

Figure 2.1 Regulation of thyroid secretion

(Modified from Tortora and Derrickson, 2006)

Biosynthesis of thyroid hormones

Thyroid hormones are synthesized in the follicular cells of the thyroid glands as shown in figure 2.2 . The steps of thyroid hormone synthesis are including:

1. Synthesis of thyroglobulin at the endoplasmic reticulum in follicular cells. Thyroglobulin is a large glycoprotein, and each molecule contains 115 tyrosine residues. Its molecular weight is about 660,000 Da. After synthesis, thyroglobulin is transported to the colloid part by the exocytosis.

2. Uptake of iodide into follicular cells. Iodide is imported into the follicular cells by Na^+/I^- symporters, which the energy being provide by the Na^+/K^+-ATPase. In the cytosol, iodide is altered to iodine by the oxidation reaction that requires hydrogen peroxide (H_2O_2) and thyroperoxidase as the cofactors.

3. Iodination of thyroglobulin. Tyrosine is iodinated at position 3 on the ring, forming monoiodotyrosine (MIT). The tyrosine molecule may be also iodinated on position 3 and 5 to form diiodotyrosine (DIT). After that, MIT and DIT or two molecules of DIT are coupled to form T_3, T_4, respectively.

4. Secretion of thyroid hormone. The thyroglobulin molecule which comprise of T_3 and T_4 is taken up into the follicular cells by endocytosis, and it is lysed by proteolytic enzymes in lysosomes before T_3 and T_4 are secreted into the plasma.

T_3 and T_4 are water insoluble. Thus, they are attached to plasma protein for transport to target organs. About 70% of circulating thyroid hormones is bound to a major binding protein, thyroxine binding globulin (TBG), 10-15% is bound to another thyroid binding protein, transthyretin (TTR), 15- 20% is bound to thyroxine binding prealbumin (TBPA), and 3% is bound to lipoproteins (Bern *et al*., 2004). L-thyroxine is indicated for the treatment of hypothyroidism in all species which may also be known as T_4 thyroxine sodium. In dogs, peak plasma concentration occurs 4-12 hours after administration and the serum half-life is approximately 12-16 hours (Plumb, 1991).

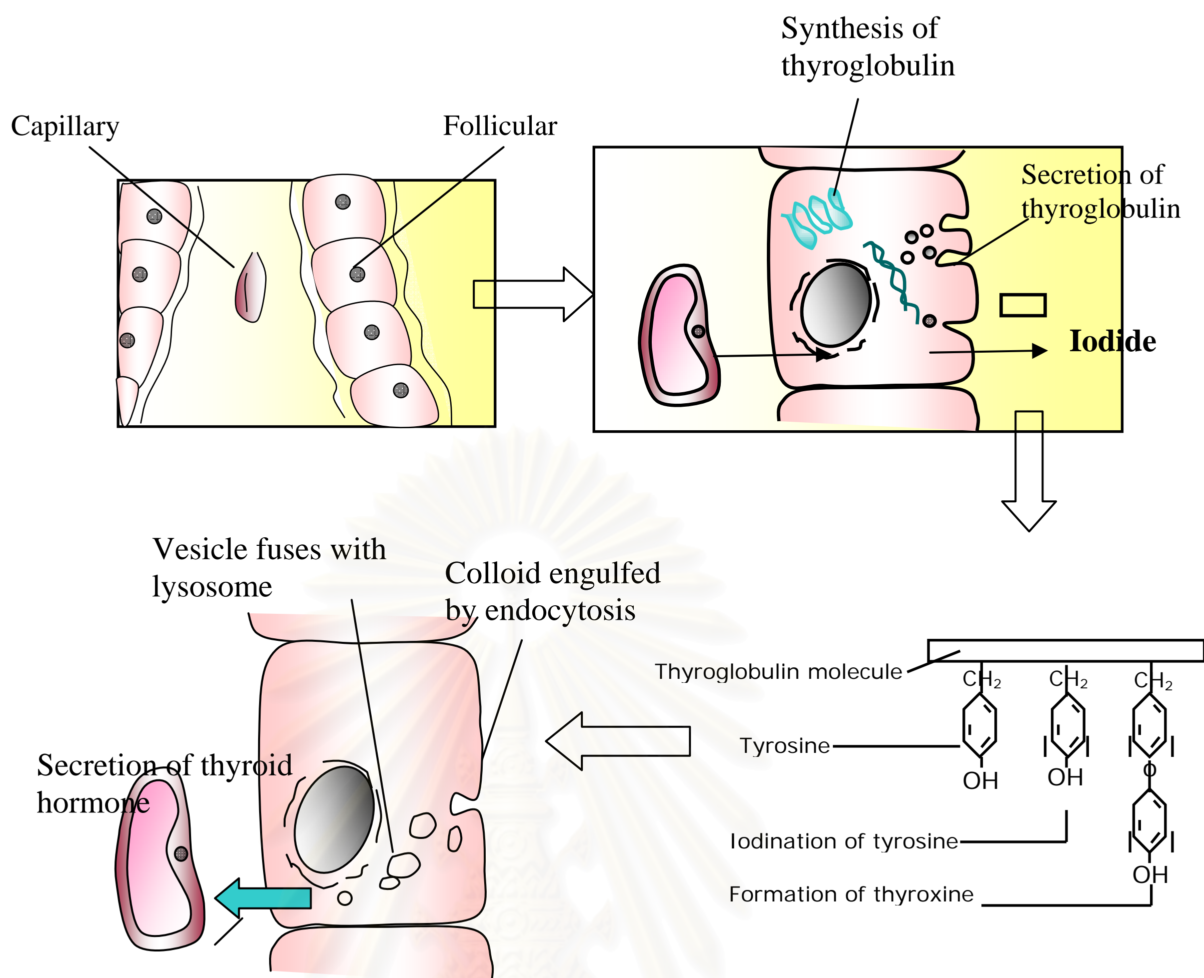

Figure 2.2 Biosynthesis of thyroid hormones (Modified from Guyton and Hall, 1996). Thyroid glands comprise capillary, follicular cells, and colloid which is located in lumen of the follicle. When thyroid glands are activated by TSH, (1) thyroglobulin is synthesized on the rough endoplasmic reticulum of the follicular cells and moved into the lumen of the follicle. (2) Iodide is taken up into follicular cells by sodium iodide symporters before it is transferred into the follicular lumen. (3) T_4 and T_3 were synthesized by iodination of iodine, and they are bound to the thyroglobulin molecule. (4) These conjugated molecules were moved back into the follicular cells by endocytosis before fused with lissome to release T_3 and T_4 in the circulation.

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Intracellular signaling

Free plasma T_3 and T_4 enter the cells through the plasma membrane and bind to their intracellular receptors. The thyroid receptor can bind to the retinoid x receptor (RXR) as a monomer, homodimer or heterodimer (Evans, 1988).

In 1994, Glass has reported that the biological effects of thyroid receptor binding by the unoccupied receptor versus the occupied receptor are different. In general, no binding of thyroid hormone receptors to DNA lead to repression of transcription whereas binding of the thyroid hormone receptor complex activates transcription as shown in figure 2.3.

One part of this corepressor complex has histone deacetylase activity (HDA) and causes a closed chromatin configulation on genomic DNA that represses basal transcription of the target gene which is associated with formation of a compact, "turned-off" conformation of chromatin (Nagy *et al*., 1997). In the presence of T_3, a conformational change within the thyroid receptor allows release of the co-repressors molecules and recruitment of a co-activator complex, and is competent to bind a group of coactivator proteins. The coactivator complex contained histone transacetylase (HAT) activity imposes an open configuration on adjacent chromatin. This open structure is thought to facilitate the assembly of the basal transcription machinery, and increases the rate of mRNA transcription of thyroid responsive genes (Torchia *et al*., 1997)

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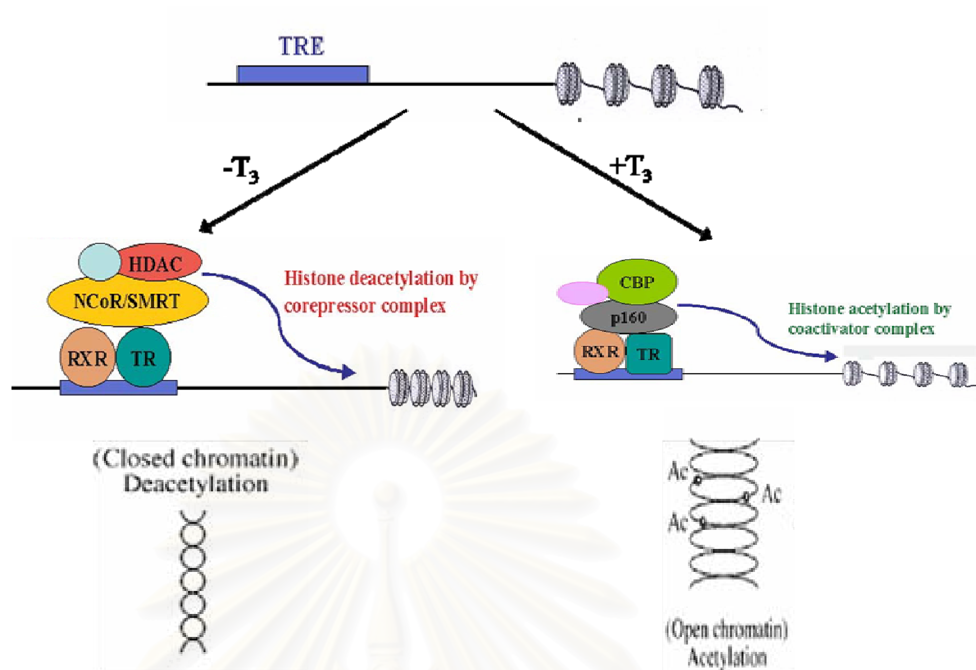

Figure 2.3 Model of activation and repression of the thyroid hormone receptor (Modified from Malik and Hodgson, 2002).

Thyroid hormone metabolism

T_3 is the thyroid hormone with the greatest biological activity, but T_4 is quantitatively secreted at much higher levels. T_4 is a prohormone that requires deiodination for convertion to T_3 (Hassi *et al.*, 2001). There are several types of enzymes that regulate thyroid hormone metabolism e.g., 1 5'-deiodinase (D1) for change T_4 to rT_3 and T_3. Similary, this enzyme acts on T_4 for alteration to T_3 in the liver and kidneys of cats, but this enzyme is not expressed in the thyroid glands of cats which differ from humans, dogs, rats, mice and guinea pigs that show strong expression of enzymes. However, this enzyme can be inhibited by propylthiouracil similar to other mammals (Foster *et al.*, 2000). Additionally, 2 5'- deiodinase (D2) is another enzyme for conversion of T_4 to T_3. In human, both types, D1 and D2, are located in the pituitary gland. Beside, D1 is also expressed in the thyroid gland, liver, and kidneys. Finally, 3 5'- deiodinase (D3), which plays role on the conversion of T_4 to rT_3, is found in the brain, skin and placenta. However, this catalyzed form rT_3 is an inactive metabolite.

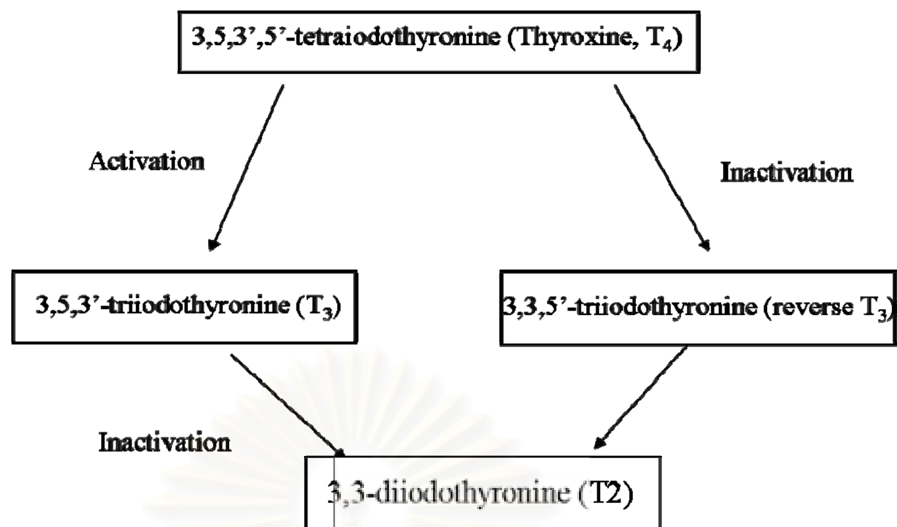

Figure 2.4 Interactions between the principle iodothyronines.

Hyperthyroidism in cats

Hyperthyroidism is a common disease in middle and older aged of cats, with prevalence of 0.33% (Lécuyer *et al*., 2006). This disease occurs by an excessive production of tetraiodothyronine and triiodothyronine. 98% of sick cats result from a functional benign adenomatous hyperplasia of the thyroid gland. Besides, food and environmental causes also have been suspected (Martin *et al*., 2000).

Diagnosis of hyperthyroidism in cats is need to considering of clinical signs and physical examination. Usage of only serum thyroid hormones are not recommended because of fluctuation of serum levels in hyperthyroidism (Peterson *et al*., 1987). The majority of sick cats may have abnormalities on routine hematology such as increases in packed cell volume, mean corpuscular volume, red blood cells count and hemoglobin concentration. Blood chemistries also show abnormalities e.g., increases in alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase and aspartate aminotransferase. The most commonly seen manifestations of the disease are significant weight loss, loss of the normal hair coat luster and patchy hair loss, normal to increased appetite and hyperactivity.

There are three options for the treatment of hyperthyroidism in cats: medical management therapy, surgical thyroidectomy, and radioactive iodine therapy, resulting in the destruction of active thyroid tissue. Drugs for treatment of the disease

are propylthiouracil (PTU), methimazole (MMI) and carbimazole (CBZ). These drugs act as inhibitors of thyroid peroxidase-catalyzed reactions which MMI has a good oral bioavailability (half life is 4-6 hours). To manage the over activity of sympathetic system, hyperthyroid cats should receive beta adrenergic antagonists to reduce tachycardia, polypnea, hypertension and hyperexcitability (reviewed by Mooney, 2001).

The phenomenon from hyperthyroidism contributes to elevate protein productions may be due to T_3 activation of protein synthesis. Several studies have shown that thyroid hormones can increase both number and sensitivity of beta adrenergic receptors (Bilezikian and Loeb, 1983; Ratge *et al*., 1985; Tse *et al*., 1980). Iwata and Honda (2004) concluded that isoprenaline and acetylcholine induced relaxations were significantly greater in hyperthyroid rats than in the control. These results indicate that acute hyperthyroidism significantly enhances β adrenergic receptor mediated relaxation. In addition, thyroid hormones may increase β adrenergic receptor numbers without alteration of affinity. Beside, thyroid hormones may also increase plasma cAMP levels and leukocyte cAMP-phosphodiesterase in humans (Andersson *et al*., 1983). Moreover, a significant decrease in receptor density was found in thyroidectomized patients when compared pre-and post-operatively (Cognini *et al*., 1983).

B. Adrenergic receptors

Types and localizations of adrenergic receptors (Jenkinson, 1973; Reiter, 2004)

There are different subtypes and localization of adrenergic receptors in each tissue as shown in the following information.

1. β adrenergic receptors

β_1 adrenergic receptors are predominantly present in cardiac tissue. β_2 adrenergic receptors are found in the heart, smooth muscles of vessels and bronchi, and presynaptics of neurons. β_1 adrenergic receptors are present in the synaptic cleft, whereas β_2 adrenergic receptors tend to be located far from adrenergic nerve terminals. For this reason, noreadrenaline are mainly a neurotransmitter for activation

to β_1 adrenergic receptors, but β_2 adrenergic receptors were activated by circulating adrenaline.

2. α adrenergic receptors

α_1 adrenergic receptors located in the synaptic region and response to neuronally released catecholamine. Vascular smooth muscle is contracted when it is activated to maintain blood pressure. α_2 adrenergic receptors are predominantly located in nerve terminals. Activation of α_2 adrenergic receptors causes a decrease in adrenaline release and more responsive to circulating catecholamines.

Distribution of adrenergic receptors in non cardiac tissues

Adrenergic receptors are widely distributed in human non cardiac tissues such as; (Motulsky and Insel, 1982)

1. Platelets

Identification of α adrenergic receptors by radioligand technique found that α adrenergic receptors in platelets are α_2 subtypes which each platelet has 200-300 α_2 adrenergic receptors. Platelets are activated to form aggregation by stimulation of α adrenergic receptors. The mechanisms of α adrenergic stimulation contribute to an inhibition of adenylate cyclase (decreases cyclic AMP) and an enhancement of calcium influx.

2. Granulocytes and lymphocyte

From principle of radioligand binding techniques for identification of receptor, it has been shown that β adrenergic receptors are present in mononuclear cells, MNC (e.g., T cells, B cells and monocytes) which predominant β_2 subtype of β adrenergic receptors (Brodde *et al*., 1986). β_2 adrenergic receptors in mononuclear leukocyte were used for investigation of alteration of β_2 adrenergic in humans because there is relation between alterations of this MNC adrenergic receptor and other tissue adrenergic receptors. Therefore, changes in β_2 adrenergic receptors measured in circulating MNC may reflex changes in the density and functional of adrenergic receptors in other tissues (Brodde *et al*., 1986).

3. Adipocytes

Adipocytes contain several types of adrenergic receptors. Stimulation of β_1 adrenergic receptor in this tissue contributed to lipolysis mediate increase of cAMP. On the other hand, reductions of lipolysis mediate inhibition of adenylated cyclase acting through the stimulation of α_2 adrenergic receptors, and an increase in phospholipid turnover mediate through activation of α_1 adrenergic receptor.

Table 2.1 Adrenergic receptor classification (Modified from Katzung, 1998)

Receptor type	Predominant Location	Predominant Effect	Predominant Agonist
β_1	heart, juxtaglomerular apparatus, synaptic cleft	increases HR, increases contractility	norepinephrine
β_2	bronchi, uterus, skeletal muscle, extra-and presynaptic, peripheral vasculature	vasodilatation, bronchodilatation	epinephrine
α_1	vascular smooth muscle, dilator muscle of iris, within synaptic cleft	vasoconstriction	norepinephrine
α_2	extra synaptic, presynaptic	vasoconstriction	Epinephrine

Table 2.2 Effector system of adrenergic receptors (Modified from Katzung, 1998)

Adrenergic receptor	G protein	Examples of some biochemical effectors
β_1	Gs	↑ adenylyl cyclase ↑L-type Ca^{2+}channels
β_2	Gs	↑adenylyl cyclase
α_1	Gq	↑ phospholipase C
	Gq	↑ phospholipase D
	Gq, Gi/Go	↑ phospholipase A_2
	Gq	? ↑ Ca^{2+}channels
α_2	Gi 1,2 or 3	↑adenylyl cyclase
	Gi ($\beta\gamma$ subunits)	↑K^+ channels
	Go	↓Ca^{2+}channels (L-and N-type)

C. β adrenergic antagonists

Propranolol is a non selective beta adrenergic antagonist, which blocked β_1 and β_2 adrenergic receptors with equal affinity. It is the first useful agent synthesized as a prototype (Black and Stephenson, 1962). Second generation beta blockers were developed later base on the concept that a lack of β_2 adrenergic activity to reduce side effects. Recently, a third generation beta adrenergic antagonists were developed, and they possess α adrenergic blocking activity such as labetalol or carvedilol (Frishman and Halprin, 1979).

Structures of β adrenergic agonists and antagonists are similarly for competitive binding with β adrenergic receptors, and catecholamines are ligands for these receptors. When these ligands bind to receptors, they generate positive chronotropic and inotropic actions. β adrenergic antagonists prevent these effect by competing with catecholamines for binding with β adrenergic receptors. When receptors are blocked, heart rate and cardiac contractility were decreased.

Short term administration of β adrenergic antagonists decreases cardiac output and blood flow whereas peripheral resistance is increased due to the blockade of β_2 adrenergic receptors. Long term administration of β adrenergic antagonists causes a return of peripheral resistance to initial values (Mimran and Ducailar, 1988). Although, the majority of these drugs are treatment for patients with cardiovascular diseases, they also have effects on blood coagulation supported by recent several studies (Hoppener *et al*., 2004; Ingram and Jones, 1966; Von Känel *et al*., 2002b).

Pharmacokinetics of β adrenergic antagonists

Metabolism

There are different metabolisms of each drug such as propranolol and metoprolol eliminated by hepatic metabolism, tend to be lipid soluble, and have short half lives. Some drugs are predominantly eliminated unchanged by renal mechanisms (e.g. nadolol), and tend to be water soluble with longer half life. The major enzyme system for metabolite these drugs is the cytochrome P450 mono-oxygenase. However, the metabolic products are less active than the parent compound, or are inactive.

Lipid solubility

Lipid solubility is different among agents, such as lipid solubility of carvedilol (Petrikova *et. al*., 2002) and propranolol is higher than atenolol and nadolol. High lipid solubility leads to a larges volume of distribution and better CNS penetration.

Adverse effects

Common side effects of β adrenergic antagonist administration are general fatigability and lethargy, anorexia, nausea, diarrhea and cold extremities. Some time, bradycardia is also occurred in β adrenergic antagonist administration because these drugs may depress sinus node activity or AV nodal conduction. However, bronchoconstriction is also less likely with β_1 selective agent.

Specific agents

Propranolol

Propranolol is a nonselective beta that blocks equal affinity for β1 and β2 adrenergic receptors, and it is highly lipophilic. Distribution of propranolol in humans (Reiter, 2004) is 4 L/kg which differs from cats that have 1.65 L/kg (Jacobs *et al*., 1997). Clearance of this drug was affected by hepatic blood flow, liver disease and administration with other drugs that affect hepatic metabolism. In cats, propranolol reduced hepatic blood flow via β blocker properties which reduce its own clearance. Additionally, hyperthyroidism is a common in the cats (Mooney, 2001) which interferes pharmacokinetics of propranolol by alteration of hepatic enzyme expression (Rubenfeld *et al*., 1978). Thus, oral bioavailability may be increased. Besides, peak serum and area under the curve of propranolol may be increased but body clearance is decreased. In human, propranolol has a relatively short half life, 3 to 6 hours (Reiter, 2004). However, half life of propranolol is shorter in normal cats, about 51 minutes, and it is even shorter when cats have high thyroid hormones, about 30 min (Jacobs *et al*., 1997).

Carvedilol

In human, carvedilol is one of a third generation non selective beta adrenergic antagonist. It blocks β_1, β_2 and α_1 adrenergic receptors. It is lipophilic, hepatically metabolized, and has active metabolites. Carvedilol possesses a

vasodilating effect mediated by blocking α_1 adrenergic receptors. Carvedilol and its metabolites have antioxidant activity and antiproliferative which may reverse endothelial dysfunction. Besides, it also has effects on Ca^{2+}, Na^+ and K^+ channels, but significances of these are unknown. The plasma half life of carvedilol is 7 to 10 hours (Reiter, 2004). However, pharmacokinetics of carvedilol in healthy cats and hyperthyroid cats has never been investigated.

Table 2.3 Adrenergic profile of selective β adrenergic antagonists (Reiter, 2004)

Drug	Brand name	β_1 Potency	Relative β_1 selectivity	α Blockade
Propranolol	Inderal ®	1.0	0	0
Carvedilol	Coreg®	10.0	0	+

Table 2.4 Pharmacokinetic profile of selected β adrenergic antagonists (Reiter, 2004).

Drug	Bioavailability (%)	Absorption (%)	Protein binding (%)	Lipid solubility	Clearance Half-life
Propranolol	30-40	>90	90-95	+++	3-6
Carvedilol	25-35	>80-90	98	++	7-10

D. Coagulation system

Blood coagulation is a basic physiological defense mechanism to prevent blood loss following vascular injury. In all species, the basic mechanism of clot formation is similar (Gentry, 2004) as shown in figure 2.5.

The coagulation system consists of three pathways as the following.

1. Extrinsic pathway

When endothelium is damaged, tissue factor exposed on the plasma membrane where it interacts with circulating FVII, or its activated form (FVIIa), to form the enzymatically reactive TF-FVIIa complex (Gentry, 2004). This complex activates FX to an active form (FXa) for production of prothrombinase complex that consists of FXa, phospholipids, calcium and FVa. FV is induced by thrombin to FVa. Prothrombinase complex acts on factor II (prothrombin) to form thrombin (Orfeo *et a*l., 2004). Thrombin can convert fibrinogen to fibrin for clot formation, or form thrombus around the area of vascular damage.

2. Intrinsic pathway

2.1. Exposure of the blood to vascular wall collagen alters factors in the blood. When FXII is disturbed, it takes on a new molecular configulation that converts it into FXIIa.

2.2 The activated FXII acts enzymatically on FXI, and changes it to active form. This reaction also requires high molecular weight kininogen (HMW) and is accelerated by prekallikrein.

2.3 The activated FX combined with FVIII on the activated platelet membrane, which becomes the major activator of FX.

3. Common pathway

FXa combines with FVa on the activated platelet membrane surfaces at specific receptor sites, and this FVa – FXa catalyst converts prothrombin to thrombin, thereby setting into motion the final cloting process, as described earlier.

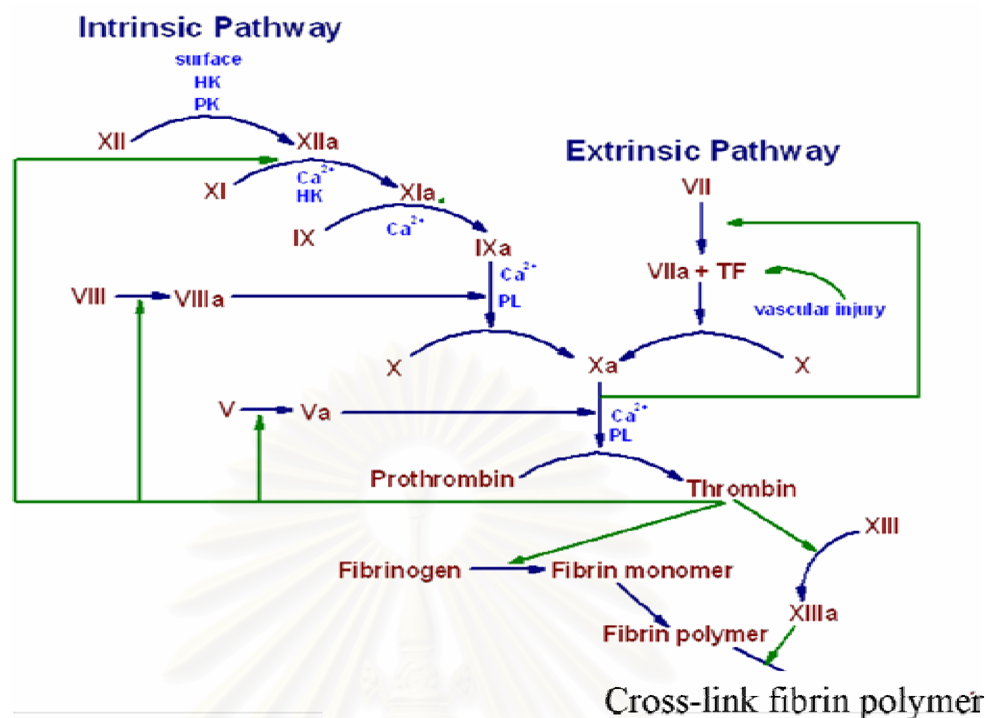

Figure 2.5 Schematicdiagram of the coagulation cascade (Modified from Dahlbäck *et al*., 2005)

E. Coagulation disorders in thyroid diseases

Coagulation disorders occur in patients with thyroid diseases that there is a great contrast between hypothyroidism and hyperthyroidism. In hypothyroidism, patients are at particular risk of hemorrhage but patients with hyperthyroidism display a tendency to develop thromboembolic complications, with major embolism accounting for up to 18% of deaths in patients dying from thyrotoxicosis (Hofbauer and Heufelder, 1997).

Pathogenesis of coagulopathies in thyroid diseases associated with synthesis and action of coagulation factors by thyroid hormones may elevated hepatic protein synthesis, and alters blood viscosity by excessive fluid loss resulting from increased metabolic rate, respiratory rate, and sweating that may contribute to the hypercoagulable state.

Table 2.5 Coagulation disorders associated with hyperthyroidism (Hofbauer and Heufelder, 1997)

	Parameter
Hypercoagulable state	- Increased metabolic rate
	- Excessive fluid loss
	- Elevated hepatic protein synthesis
	- Enhanced thrombin and plasmin activity
	- Cardiogenic thromboembolic disease

Patients with hypothyroidism exhibit minor bleeding tendency, which may manifest as menorrhagia or easy bruising. Disorders of blood coagulation in hypothyroidism were found combination with von Willebrand's disease, and this result contributes to a decreased activity of factor VIII/von Willebrand factor complex. This phenomenon causes an increase in factor VIII activity, and is confirmed by an elevation of activated partial thromboplastin time, APTT (Reviewed by Hofbauer and Heufelder, 1997).

Table 2.6 Coagulation disorder associated with hypothyroidism (Hofbauer and Heufelder, 1997).

	Parameter
Coagulation factors	- Decreased activity of factors VII, VIII coagulant activity, IX, XI, XII
	- Increased biological half life of factor II, VII, IX, X

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Hyperthyroidism increases sensitivity of adrenaline on β_2 adrenergic receptors because it increases protein synthesis that implies β_2 adrenergic receptors related mechanism for increases in factor VIII levels. The observation was found that adrenaline can enhance factor VIII levels via β_2 adrenergic receptors that are couple

with cAMP. Indeed, a rise in intracellular cAMP following β adrenergic stimulation via the adenyl cyclase system is sufficient stimulus to induce acute release of factor VIII without a concomitant increase in intracellular calcium (Von Känel and Dimsdale, 2000), and factor VIII activity may rise so quickly after adrenaline infusion. There are several possible mechanisms which appear to be an activation of intravascular material release from storages or new synthesis. Moreover, this activation may be due to an increase in the number of available active sites when the normal factor VIII molecule is splitted into smaller subunits (Ingram *et al*., 1977). However, the rise of adrenaline in the modulation of factor VIII activity in the hyperthyroid state remains unclear.

Relation of hyperthyroidism with factor VIII levels and thrombosis.

Ingram and Jones (1966) studied in healthy volunteers who were assigned to receive adrenaline by intravenous injection, and they found that factor VIII activity could rise so quickly. In patients with hyperthyroidism, proteins were increased. This is due to an enhancement of β adrenergic receptor number (Tse *et al*., 1980). The results of this study contribute to an increase response of adrenaline, and an induction of increased factor VIII levels may imply a β_2 adrenergic receptor related mechanism. Because of peak responses of hemostasis variables to adrenergic agents occurred within 15 to 40 min; thus, new synthesis of factor VIII appears unlikely. Rather preformed and stored molecules must be released into circulation. Adrenaline infusion induces short term recruitment of functionally active factor VIII from the spleen. FVIII, vWF and t-PA, all are stored in endothelial cells within Weibel-Palade bodies of the Golgi apparatus (Von Känel and Dimsdale, 2000). Additionally, Von Känel *et al.* (2002a) conclude that adrenergic stimulation of the vascular endothelial β_2 adrenergic receptor underlies increased t-PA and clotting factor VIII activity, by releasing these molecules as well as hemostatically active large von willebrane factor multimer from endothelial storage sites into the circulation. A hight plasma level of factor VIII is a risk factor for venous thromboembolism (Kyrle *et al*., 2000), and this observation was confirmed by another study from O' Donnell *et al*. (2001) who reported that increases in plasma FVIII levels (> 150 IU/dl) is an important risk factor for venous thromboembolic disease (VTE) in humans. Height FVIII levels are present in 25% of patients with confirmed VTE, and are associated with a 5-8 fold

increased risk of VTE. On the contrary, indirect evidence has come from studies in haemophilia (low factor VIII, normal vWF and normal platelet function) which showed a 80% reduction in mortality from coronary heart disease (Rosendaal *et al*., 1989).

In 1995, Mischke *et al*. investigated the activity of the coagulation factor VIII in cats by using an optimized test, reference ranges were derived from 58 healthy cats. They found that the factor VIII activity in cats was 13 fold higher than in humans. This result may be an important clue of thrombosis in cats. However, there are no study which determine relation of factor VIII with hyperthyroidism in cats. The clinical records of 44 cats with distal aortic thromboembolism were investigated. The mean age was 8.7 years (age range 2-16 years), and cats with thromboembolism have the median survival time of 6 months (Schoeman, 1999). Preventative treatment such as aspirin (Green, 1985) and warfarin (Harpster and Baty, 1995) have been recommended. Recurrence of thromboembolism has been reported in 75% of cases on aspirin therapy in a study (Pion and Kittleson, 1989), wheres another study reported five cats surviving more than 8 months on aspirin therapy without recurrence of thromboembolism (Flander, 1986). Furthermore, warfarin prophylaxis had a 43% recurrence rate, and was associated with complication such as bleeding episodes (20%) and sudden death (12%) (Harpster and Baty, 1995).

In recent years, incidences of venous thrombosis in patients with hyperthyroidism and high factor VIII levels become excessive adrenergic activity occurring in hyperthyroid patients, and could have a direct effect on the production of factor VIII. Therefore, it seems clear that β blockers can prevent thrombosis from high factor VIII without severe bleeding complication.

F. Effect of beta blockers on thrombosis

The administration of adrenaline contributes to a rapid increase in the plasma level of clotting factor VIII in human subjects (Egeberg, 1963b; Ingram, 1961). Similarly, Ingram and Jones (1966) investigated the rise in clotting factor VIII induced by adrenaline, and found that the increase in clotting factor VIII induced by adrenaline was blocked by propranolol but not phentolamine. This indicated that the effect of adrenaline on factor VIII was mediated by β adrenergic receptor (Ingram and Jones, 1966). However, these results are disagreed by the finding of Gardikas *et al*.

(1968) who found that β adrenergic antagonists do not inhibit the effect of adrenaline on the factor VIII activity in humans. In 1982, Roger and Shane. investigated the effect of thyroid hormones and propranolol on factor VIII activity. They found that factor VIII activity increased by thyroid hormones was blocked by propranolol. Moreover, propranolol can decreased factor VIII levels in patients with history of deep vein thrombosis and high factor VIII levels (Hoppener *et al*., 2004).

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CHAPTER III

MATERIALS AND METHODS

A. Animals

The use of animals in this experiment was approved by the Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University. The experiment was performed on healthy, mixed-breed cats of both sexes with body weights between 2-5 kg. Blood collection was performed, and the hematology and blood chemistry profile were routinely determined. Before starting the experiments, cats were checked up for complete blood count, blood parasites, liver and kidney function tests, feline immunodeficiency virus antibody, and feline leukemia virus antigen. The animals were vaccinated to prevent rabies, feline panleukopenia, feline herpes virus, and feline calici virus. All animals were housed individually, and body weights were measured weekly. The animals were fed with standard cat chow (S.W.T. co., Ltd, Samut Prakan, Thailand) following the recommendation of the company and water was given *ad lib*.

B. Experimental protocols

The experimental animals were randomly divided into two groups, as shown in figure 3.1.

Group 1 (Control group)

Period 1	Period 2	Period 3	Period 4	Period 5
Standard cat chow	Beta blocker 1	Washout period	Standard cat chow	Beta blocker 2
0	14	28	42	56

70 Days

Group 2 (hyperthyroid group)

Period 1	Period 2	Period 3	Period 4	Period 5
Thyroxine 1	Thyroxine 1+ Beta blocker 1	Washout period	Thyroxine 2	Thyroxine 2 + Beta blocker 2
0	14	28	42	56

70 Days

Figure 3.1 Diagrams of the experimental protocol.

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The control group: Four normal adult cats were used. The first period, all cats were received standard cat chow without drugs for 14 days, then followed by receiving beta adrenergic antagonists, either propranolol, Betalol® or carvedilol, Caraten® (Berlin Pharmaceutical Industry Co., Bangkok, Thailand) administered by orally at the dose of 1 mg/kg/day for 14 days (period 2). All cats in this group were randomly selected to treat first with either propranolol or carvedilol. Therefore, half of cats received propranolol first and another half of cats must receive carvedilol. After that, animals were allowed to rest for 14 days for washout of the previous substance (period 3). After 14 days of the washout period, cats were received nothing for 14 days (period 4); then switched to receive another beta adrenergic antagonist for 14 days (period 5).

The hyperthyroid group: Eight adult cats were used. On the first period, all cats were received 50 μg/kg/day once daily of thyroxine, Eltroxine™ (Glaxo Wellcome GmbH & Co. Bad Oldesloe, Germany) in the form of 100 μg thyroxine formulated as an effervescent tablet (period 1). For the second period, half of animals were randomly selected to receive thyroxine together with beta adrenergic antagonists, either propranolol or carvedilol for 14 days. After that, animals were allowed to rest and washout for 14 days (period 3), and then they were received thyroxine for 14 days (period 4) followed by thyroxine together with another beta adrenergic antagonist for 14 days (period 5).

At the end of each period all cats were anesthetized with tiletamine /zolazepam (Zoletil®; Virbac Laboratories, Carros, France) at the dose of 25 mg/cat subcutaneously for blood collection from the external jugular vein using a 10 ml plastic syringe and a 21G needle. Blood samples were drawn between 10.00 and 12.00 a.m., separated into three tubes contained nothing for serum total T_4 assay, 3.2 % sodium citrate for factor VIII activity, PT, and APTT assay, and heparin for mononuclear cell isolation.

C. Methods

1. Measurement of total T_4, hematology and blood chemistry

Total T_4 were analyzed by a competitive chemiluminescent enzyme immunoassay (Immulite, Diagnostic Products Corporation offices, Los Angeles, USA). EDTA blood was used to assess the complete blood cell count by an

impedance cell counter (Coulter T-890, Northwell Drive, Luton, Beds, England). Biochemical parameters were determined by an automate spectrophotometer (Humalyzer 2000, Humans Gesellschaft für Biochemica and Diagnostica mbH, Wiesbaden, Germany).

2. Determination of prothrombin time (PT) and activated partial thromboplastin time (APTT)

PT and APTT were determined by a coagulometer (Coag-A-Mate® XM, Organon teknika Corporation, Durham, North Carolina) that utilizes a photo-optical clot detection technique based on the principle that light passing through plasma is scattered as fibrinogen that is converted to fibrin. Light transmitted through the plasma sample is measured by photo detection. As a clot form, light scattered by the plasma sample increases, and transmitted light falling on the photo detector decreases correspondingly.

2.1 Prothrombin time (PT)

Prothrombin time (PT) is the time for activated clotting factors until initiated clot formation. This assay is used as a screening test to measure the extrinsic and common pathways of the clotting process in the blood. Plasma samples for PT measurement were prepared by centrifugation of citrated blood at 2,500xg for 15 minutes. One hundred μl of this plasma were transferred to a cuvette for warming approximately 20 minutes before beginning the test. The clotting reaction was initiated when 200 μl of Simplastin® Excel were added (Biomerieux, Inc., Murcy I' Etoile, France). During this time, sample and reagent were complete mixing. Clot formation was determined by measuring the rate of change of light transmission through the sample by the coagulometer.

2.2 Activated partial thromboplastin time (APTT)

For APTT measurement, plasma samples were prepared as described in the PT measurement. The clotting reaction was initiated when the Platelin LS reagent (Biomerieux, Inc., Murcy I' Etoile, France) and $CaCl_2$ were added. This cycle was spending 5 minutes to complete.

3. Determination of factor VIII activity in plasma

3.1 Reagents

The comatic® Factor VIII kits (Chromogenix Instrumentation Laboratory S.p.A. Milan, Italy) contain Chromogenic substrate (N-a-Z-D-Arg-Gly-Arg-pNA) (7.7 mg), factor reagent (Bovine factor IX, factor X, thrombin colyophilized with $CaCl_2$, phospholipids) and Coamatic factor VIII buffer (Tris 0.0025 mol/L; pH 7.9, 1% BSA, NaCl).

3.2 Measurement principles

In the presence of calcium ions and phospholipids, factor X is activated to factor Xa by factor IXa. This activation is greatly stimulated by factor VIII which acts as a cofactor in this reaction. By using optimal amounts of Ca^{2+}, phospholipids, factor IXa, and an excess of factor VIII, factor Xa hydrolyses the chromogenic substrate S-2765 and liberates the chromophoric group, pNA. The colour is then read photometrically at 405 nm. The generated factor Xa and the intensity of colour are proportional to the factor VIII activity in the sample.

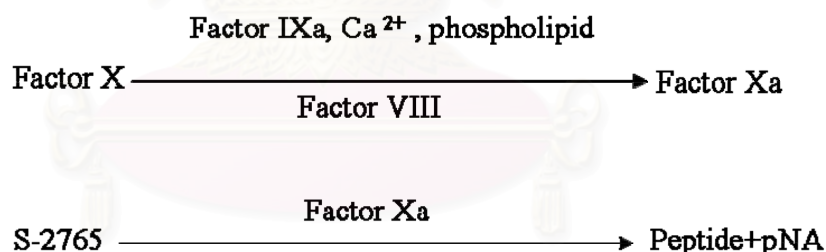

Figure 3.2 Principle measurements of factor VIII activities

3.3 Procedure

Plasma samples for this assay were separated from 3.2 % sodium citrate blood using centrifugation at 2,500×g for 15 minutes at 4°C (Universal 32/32R, Hettich zentrifugen GmbH & Co.KG, Deutschland). After that, plasma samples were prediluted by mixing 1 volume of plasma with 21 volume of Coamatic factor VIII buffer, then diluted further as followed, 25 µl sample with 2000 µl of

buffer. Fifty μl of diluted plasma were transferred to a microplate for reading the absorbance at 405 nm, using a reference wavelength of 490 nm.

4. Western blot analysis

4.1 Isolation of Mononuclear leukocytes

Mononuclear leukocytes were used because its change was shown to have relation with the changes of density and functional of β adrenergic receptors in other tissues (Brodde *et al*., 1986; Ratge *et al*., 1985). Mononuclear leukocytes were isolated from heparinized blood (6 ml) and prepared as described by the manufacturer. In briefly, heparinized blood samples were added with 5 ml of phosphate buffered saline, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) and mixed by inversion, called blood saline. Carefully layer 8 ml of the blood saline mixture was poured onto the Histopaque®-1077 Hybri –Max (Sigma chemical Co., St.Louis, Mo, USA) before centrifuged at 400×g for 30 minutes at the room temperature. The mononuclear cells were transferred from the gradient plasma to a clean tube and washed in phosphate buffered saline, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4), and these cells were centrifuged again at 250×g for 10 minutes at the room temperature. Leukocyte pellets were separated and kept at -20 C° until used.

4.2 Mononuclear leukocyte membrane protein preparation

Pellets of mononuclear leukocyte were homogenized in 50-100 μl of lysis buffer composed of 0.5 M TRIS, 10 mM EDTA, 10 % DOC, 10% NP 40, 1.5 M NaCl, 10% SDS, and protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). The homogenate was centrifuged at 4 °C for 10 min at 10,000×g (Universal 32/32R, Hettich zentrifugen GmbH & Co.KG, Deutschland). The supernatant was removed and stored at -20°C for determination of beta 2 adrenergic receptor (β_2 ADR) density with the Western blot analysis. Sample protein concentrations were determined by the Lowry method (Lowry *et al*., 1951).

4.3 Electrophoresis

Ten µl of sample buffer (1.5 M Tris, pH 6.8, 50% Glycerol, 10% SDS, 0.05% Bromophenol blue, and 2%-Mercaptoethanol) was added to a protein sample and mixed by vortex. Fifty µg of protein samples were denatured by heating to 95-100 °C and resolved by 10% sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis using a vertical minigel system (Bio-Rad laboratories, Hercules, CA, USA). Each sample was separated in triplicate.

4.4 Immunodetection for beta 2 adrenergic receptors

Subsequent to separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P®; Amersham Biosciences, Arlington Heights, IL) in Tris-glycine transfer buffer. Blotted membranes were then blocked with 5% non fat powdered milk in Tris-buffered saline for 4 hour at the room temperature. For identification of proteins, membranes were washed (2 × 5 min) and incubated overnight at 4°C with the primary antibodies diluted in 1% milk. The primary antibody was the polyclonal anti-rabbit β2 ADR (Santa Cruz Biotechnology, Inc. St.Bergheimer, Heidelberg, Germany) at the dilution of 1:1000. After thorough washing procedures with Tris-buffered saline, membranes were exposed to the horseradish peroxidase-conjugated secondary antibody (dilution of 1:10000) for 1 hr at the room temperature. This incubation step was terminated with several washes and the immunoreactive protein bands were visualized using enhanced chemiluminescence kit (ECL Plus®; Amersham Biosciences) according to manufacturer's instructions. Membranes were exposed to film (Hyperfilm-ECL®; Amersham Biosciences) for times adequate to visualize chemiluminescent bands. Blots were reprobed with 1:450,000 monoclonal anti-β-actin (secondary antibody, clone AC-15, Sigma Chemical Co.).

For calculation of band density, the Scion Image program was used (Scion Image; Scion Corporation, Frederick, MD). In briefly, the protein immunoreactive bands were obtained by a scanner and measured density by the Scion Image program in both parameters (β 2 ADR, β actin). Proportions of β 2 adrenergic receptors on β actin were calculated.

5. Measurement of protein concentration

Total protein concentrations used in Western blot analyses were measured according to the Lowry's method (Lowry *et al*., 1951) by commercial kits for the protein assay (Bio-Rad laboratories, Hercules, CA, USA).

5.1 Reagents

Reagent A is alkaline copper tartrate solution. Reagent B is dilute folin reagent. Immonoglobulin G (IgG) was used as a standard at 0, 7.3, 14.6, 36.5, 73, and 109.5 μg/100 μl.

5.2 Procedure

Samples or standards were mixed with 100 μl of reagent A and followed by 800 μl of Reagent B, and incubated for 15 min at the room temperature. The absorbance was then measured at 750 nm. A standard curve was plotted as a linear correlation for determination of the unknown protein concentration.

6. Statistical analysis

All values are expressed as mean $\pm$ S.E.M. The means of thyroxine, PT, APTT, factor VIII levels and β 2 ADR density among periods were compared by the one way analysis of variance (ANOVA) with repeated measures design. When the outcome of the ANOVA was significant, the Student-Newman-Keuls post-hoc test was used. Data in the same period were compared by the Unpaired Student's t-test using the Prism Statistical Software. Differences were considered significance at $P < 0.05$.

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CHAPTER IV

RESULTS

The results of this study were organized into six parts as follows:

1. The body weight during cats received L-thyroxine and beta adrenergic antagonists (propranolol and carvedilol).

In this study, cats were divided into two groups, including control and hyperthyroid groups. Body weights were determined weekly. In control group, there was no change of body weights during propranolol or carvedilol administration when compared with the no drug period (figure 4.1). In the hyperthyroid group, L-thyroxine alone was not affect body weight when compared with the control group. The combinations of thyroxine with either propranolol or carvedilol, the body weights were still unchanged.

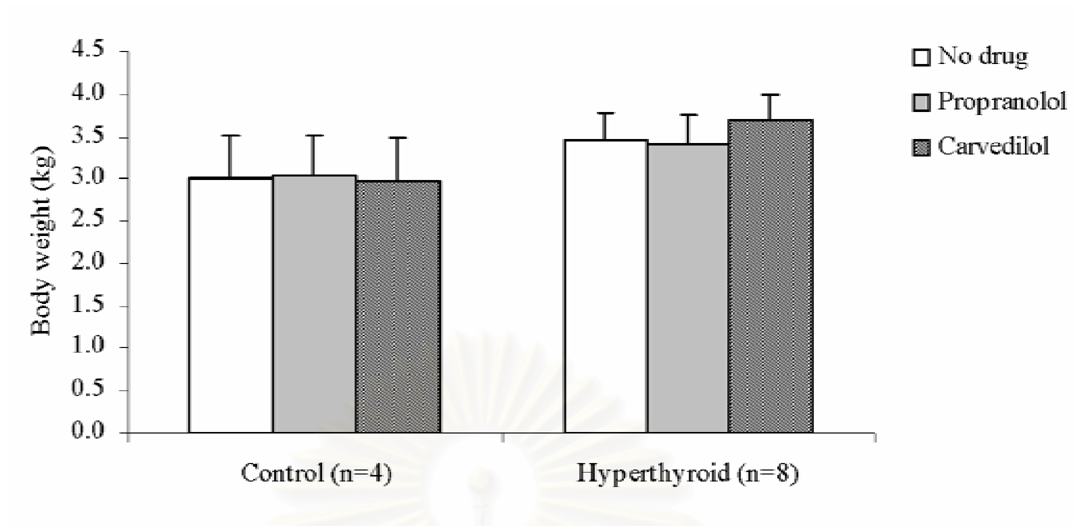

Figure 4.1 Body weights of cats in control and hyperthyroid cats. Bar graphs show body weights of cats in control and hyperthyroid groups during no drug, propranolol and carvedilol administration. Data represent mean ± S.E.M.

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2. Effects of beta adrenergic antagonists (propranolol and carvedilol) on serum thyroxine levels in control and hyperthyroid cats.

In the control group, there was no significantly difference among periods. In the hyperthyroid group, during L-thyroxine administration alone, thyroxine levels were significantly increased compared to the control group (P<0.001). Combination administration of L-thyroxine and propranolol administration caused a significant decrease in thyroxine levels from 79.32±7.90 nmol/L to 31.26±6.71 nmol/L (P< 0.01), and this serum thyroxine level did not differ from the control group receiving only propranolol. Combination of thyroxine and carvedilol in the hyperthyroid group also significantly decreased serum thyroxine levels from 79.32±7.90 nmol/L to 44.66±10.43 nmol/L (P<0.01) as shown in figure 4.2.

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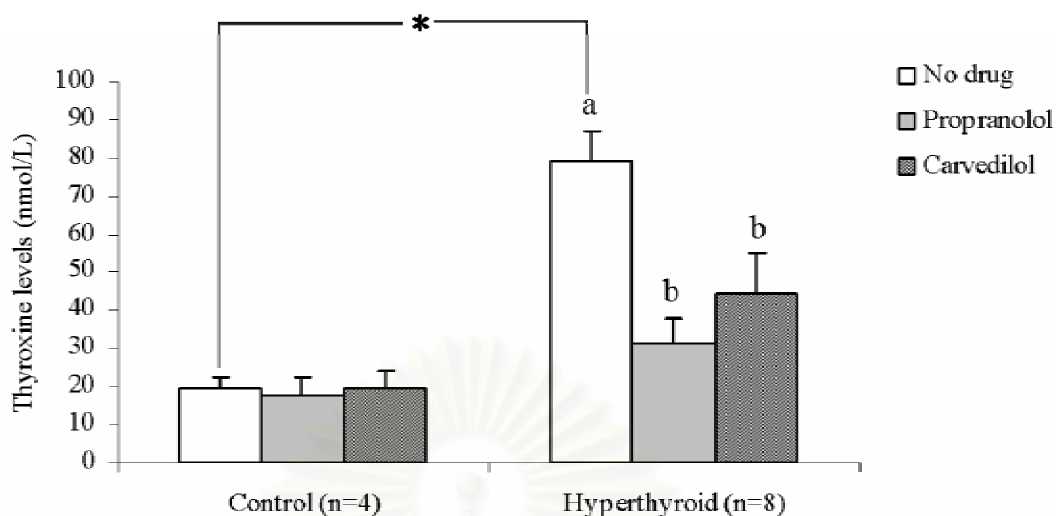

Figure 4.2 Serum thyroxine levels in control and hyperthyroid cats. Bar graphs show serum thyroxine levels in control and hyperthyroid cats given no drug, propranolol and carvedilol. The values are mean ± S.E.M. * represents a significant difference (P<0.001) between control and hyperthyroid groups by using the unpaired t-test, [a,b] represent significant differences (P<0.01) among periods in the same group by using the one way ANOVA with repeated measures design followed by the SNK post hoc test.

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3. Effects of beta adrenergic antagonists (propranolol and carvedilol) on PT values in control and hyperthyroid cats.

In the control group, there was no change of PT value among no drug, propranolol, and carvedilol periods (figure 4.3). In the hyperthyroid group, L-thyroxine alone was not affect to PT value compared to the control. The combinations of thyroxine and propranolol significantly decreased PT compared to the control group (*P*< 0.05), but the combination of thyroxine and carvedilol did not affect the PT values, and did not differ from the control.

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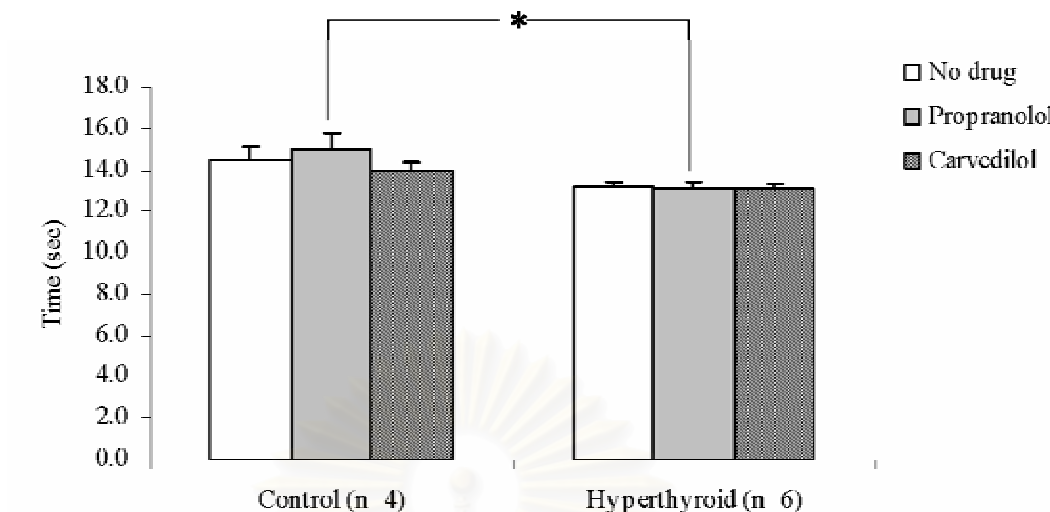

Figure 4.3 PT values in control and hyperthyroid cat. Bar graphs show PT levels in control and hyperthyroid cats. The values are mean $\pm$ S.E.M. *represents a significant difference (P<0.05) between control and hyperthyroid groups using the unpaired t-test.

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4. Effects of beta adrenergic antagonists (propranolol and carvedilol) on APTT values in control and hyperthyroid cats.

In comparison among periods in the control group, there was no significantly difference in each condition of the control group**.** During L-thyroxine administration alone, APTT value was not altered from the control group. When combined L-thyroxine with propranolol, APTT still did not change. Combination of L-thyroxine with carvedilol also did not show any significant difference compared among periods and compared to the control as shown in figure 4.4.

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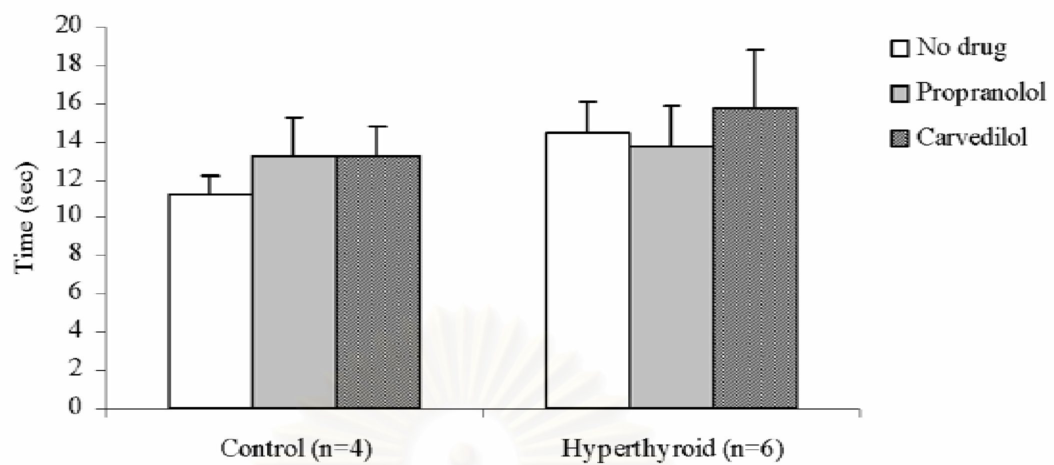

Figure 4.4 APTT values in control and hyperthyroid cats. Bar graphs show APTT values in control and hyperthyroid cats given no drug, propranolol, and carvedilol. The values are mean ± S.E.M.

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5. Effects of beta adrenergic antagonists (propranolol and caevedilol) on factor VIII levels in control and hyperthyroid cats.

In the control group, there was no significant difference during propranolol and carvedilol administration. In the hyperthyroid group during L-thyroxine administration alone, factor VIII levels were significantly increased from the control group, (6.19±0.69 IU/ml to 9.70±1.01 IU/ml, $P<$ 0.05). When combined administration of L-thyroxine and propranolol, factor VIII levels were decreased from 9.70±1.01 IU/ml to 8.94±1.07 IU/ml in comparison with the L-thyroxine treatment alone. However, both values were not significantly different. Combination administration of L-thyroxine and carvedilol significantly decreased factor VIII levels from 9.70±1.01 IU/ml to 8.39±0.80 IU/ml as compared to administration of L-thyroxine alone ($P<0.05$), shown in figure 4.5.

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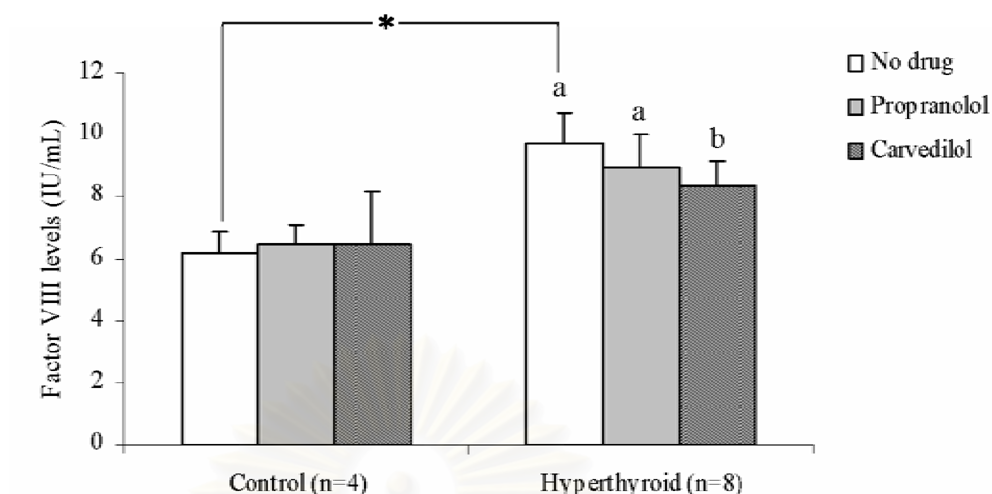

Figure 4.5 Factor VIII levels in control and hyperthyroid cats. Bar graphs show factor VIII levels in control and hyperthyroid cats given no drug, propranolol, and carvedilol. The value are mean + S.E M. * represents a significant difference (P<0.05) between control and hyperthyroid groups by using unpaired t-test, [a,b] represent significant differences (P<0.05) among periods in the same group by using the one way ANOVA with repeated measures design followed by the SNK post hoc test.

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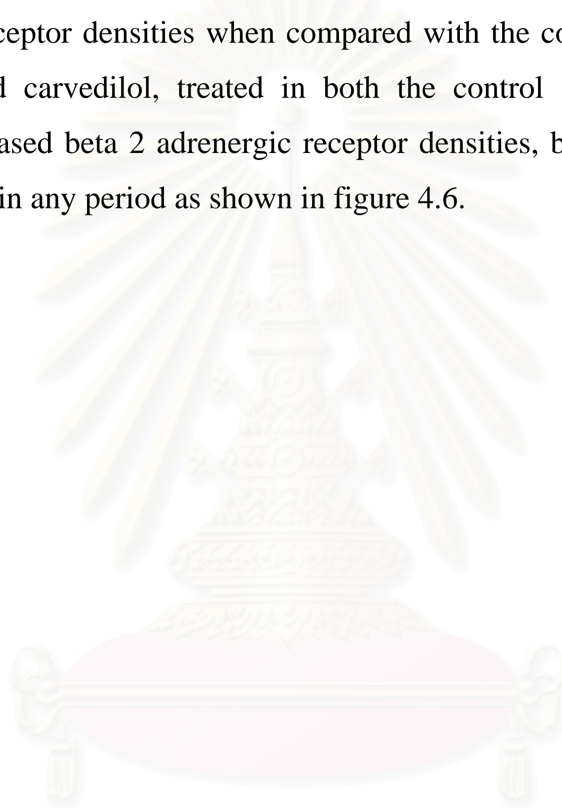
6. Effects of beta adrenergic antagonists (propranolol and carvedilol) on beta 2 adrenergic receptor densities at mononuclear leukocytes in control and hyperthyroid cats.

In the control group, during periods of propranolol and carvedilol administrations, beta 2 adrenergic receptor densities were decreased but did not reach statistical differences when compared to the no drug period.

In the hyperthyroid group, thyroxine at this dose did not increase any beta 2 adrenergic receptor densities when compared with the control group. Even though propranolol and carvedilol, treated in both the control and hyperthyroid groups, tended to decreased beta 2 adrenergic receptor densities, but statistical significances did not achieve in any period as shown in figure 4.6.



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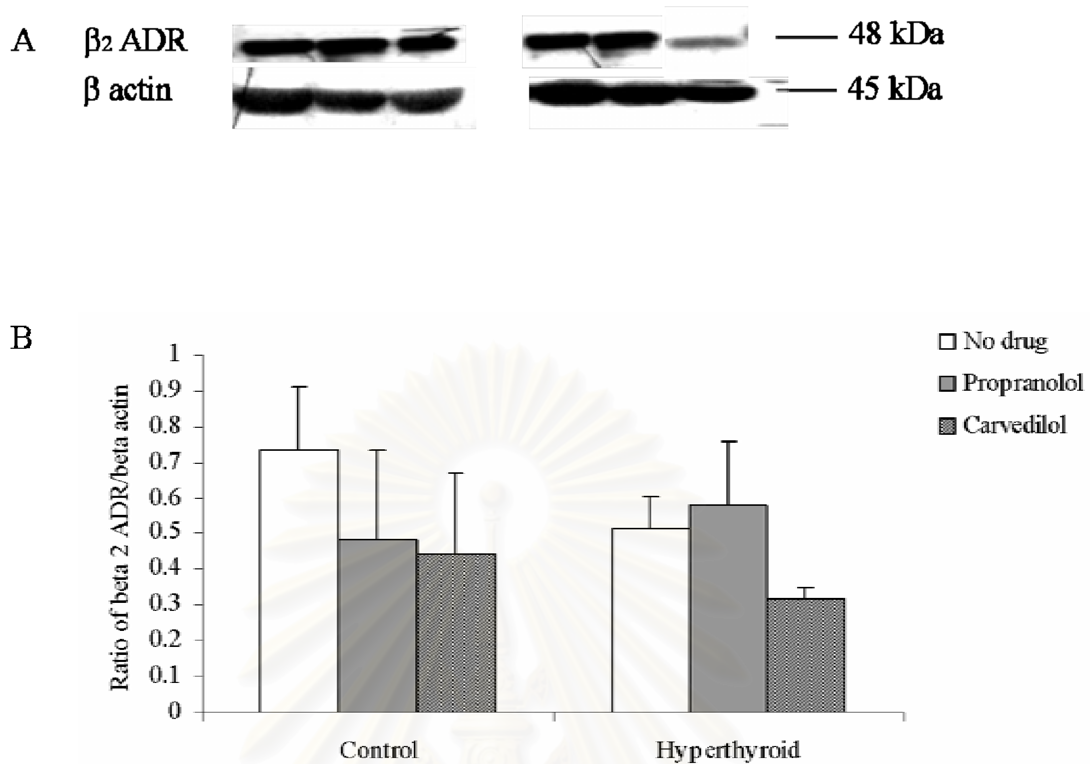

Figure 4.6 β_2 adrenergic receptor densities in control and hyperthyroid cats. (A) Photographs represents examples of immunoreactive bands in Western blot probed with specific antibody to β_2 adrenergic receptors (β2 ADR) and β-actin. (B) Histogram illustrates mean (± S.E.M) of Beta 2 ADR/β-actin protein levels of mononuclear leukocytes in control and hyperthyroid cats.

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CHAPTER V

DISCUSSION

This research attempted to assess two hypotheses. First, hyperthyroidism increased beta adrenergic receptor density and factor VIII levels in cats. Second, there were any differences between carvedilol and propranolol on beta adrenergic receptor density and factor VIII levels in cats.

The present study has confirmed and validated the model of hyperthyroidism in cats by using thyroxine supplementation. The reason that supports this model is cats given L-thyroxine have shown the significant higher levels of serum thyroxine in animals (79.32 nmol/L), and even higher than diagnostic levels (> 54.07 nmol/L) of clinical hyperthyroidism in cats reported by Willard *et al.* (1989). Propranolol and carvedilol affect to serum thyroxine levels only in the hyperthyroid group by decreasing serum thyroxine to the basal levels. The mechanism of action still is unclear, but there are two possible mechanisms that have been described. First, beta adrenergic antagonists appear to inhibit TSH-stimulated cAMP synthesis, and these result in a decline of thyroid hormone production (Nedvidkova and Stolba, 1989). Secondly, some beta adrenergic antagonists possess a membrane stabilizing effect, especially at low doses (Heyma *et al*., 1980). Carvedilol and propranolol also possess a membrane stabilizing effect, and may reduce thyroxine levels by interfering membrane function of thyroid glands. As indicated by its chemical formula, carvedilol is highly lipophilic, and it readily enters into the non-polar part of membranes and easily exerts the membrane stabilizing effect. Moreover, Carvedilol molecules have more massive molecules that propranolol, which can further extend and induce more membrane structural changes resulting in disorganization of membrane receptors and channels, as well as the activity of membrane enzymes (Petrikova *et al*., 2002). Beside, there is a direct effect of β adrenergic blockade on β_2 adrenergic receptors by decreased mRNA production of thyroid hormone receptors (Shahrara *et al*., 2000). But there was no difference between propranolol and carvedilol on thyroxine levels in our study.

We have noted significant increases in factor VIII levels in hyperthyroid cats. This result is supported by previous observations. Several authors have found

increases in plasma factor VIII levels in hyperthyroid patients (Egeberg *et al*., 1963a; Rogers and Shane, 1983). It has seemed surprising that factor VIII activity could rise so quickly on infusing adrenaline (Ingram and Jones, 1966). This evidence suggests that this change could be classified as a β_2 adrenergic effect, and is supported by the findings of Ingram (1961). They tested adrenaline, noradrenaline and isoprenaline effects on risings of factor VIII levels, but only adrenaline had produced a rise in factor VIII levels. There are several investigations about types of adrenergic activities related to factor VIII. These investigators have implied that β_2 adrenergic stimulation may have related mechanisms for increases in factor VIII levels (Ingram and Jones, 1966; Ingram *et al*., 1977; Von Känel and Dimsdale, 2000; Von Känel *et al*., 2002b). Intracellular mechanisms may lead to regulation of factor VIII release by adrenaline, and these are coupled with the phospholipase C system. This demands an increase in intracellular calcium concentration (Von Känel and Dimsdale, 2000), and β_2 adrenergic receptors may bind to Gq activity phospholipase C (Zaugg *et al*., 2002). However, increase of clotting factor VIII occurs in several conditions such as strenuous exercise, fever, pregnancy, renal failure, intravascular haemolysis (Simone *et al*., 1965), and desmopressin (DDAVP) administration, commonly used as a nonreplacement therapy for mild von Willebrand disease (VWD) and hemophilia A in humans may also increase factor VIII levels (Xu *et al*., 2004).

Several experiments have shown that β adrenergic blockade inhibits the effect of thyroid hormone on an increase in factor VIII levels. These results are in agreement with the finding of Ingram and Jones (1966), that β adrenergic blockade, propranolol, can prevent an elevation of factor VIII. Additionally, the rise in clotting factor VIII can increase the intravenous administration of the peptide hormone vasopressin (Rosenberg *et al*., 2000). Cort *et al.* (1981) suggest that vasopressin may cause the release of humoral second messengers from the central nervous system, which induce the release or increase the synthesis of factor VIII. This is supported by Xu *et al*. (2004) who suggested that Desmopressin (DDAVP) induces a rapid increase in plasma levels of both von Willebrand factor and factor VIII within 30-60 minutes due to release from Wiebel Palade bodies (WPBs) in endothelial cells. DDAVP is effective in humans and dogs, but not in mice (Xu *et al*., 2004). The stored factor VIII may be synthesized by endothelial cells, or endothelial cells take up factor VIII

from blood and store it in WPBs with VWF, and this stored factor VIII can be released after DDAVP administration. Sowers *et al.* (1976) reported data suggesting that thyrotropin-releasing hormone may have a physiologic role in the modulation of vasopressin release in humans. In our experiment, plasma levels of arginine vasopressin associated with hyperthyroidism have not been evaluated. However, it is possible that thyroid hormones may affect the level of plasma factor VIII through a yet to be defined mechanism involving the neuroendocrine system, perhaps associated with alterations in endogenous arginine vasopressin. In addition, during factor VIII levels were increased from overactivity of adrenergic system, plasma and urine levels of epinephrine and norepinephrine are normal or low in hyperthyroid patients (Landsberg, 1977). It has been suggested that thyroid hormones enhance the peripheral effects of catecholamines.

Moreover, hyperthyroidism induces a significant increase in factor VIII levels, and this corresponds with short activated partial thromboplastin time (Simone *et al.*, 1965). Our data are not agreed with finding above, but we found an increase in factor VIII levels without shorter of activated partial thromboplastin time. Similarly, Erem *et al*. (2002) found increases in fibrinogen, factor IX, and von Willebrand factor activity, but there was no change in prothrombin time (PT) or activated partial thromboplastin time (APTT). Moreover, patients may have abnormalities of blood coagulation while PT and APTT levels are in normal limits (Rennie *et al.*, 1978). In this experiment, it was surprising that APTT values were not changes by L-thyroxine, carvedilol or propranolol administration while PT values in hyperthyroid cats receiving propranolol were decreased. Therefore, it is needed further investigation.

The result of this study is indicated that carvedilol can reduce factor VIII levels but not by propranolol because the rise in clotting factor VIII was mediated by β_2 adrenergic receptors. This phenomenon was supported by investigation of Molenaar *et al*. (2005). They investigated the effectiveness of carvedilol in heart failure by determining its specific properties in human heart β_1- and β_2 adrenergic receptors, and found that carvedilol was a 13-fold more potent competitive antagonist of the effects of adrenaline at β_2 adrenergic receptors ($-\log K_B = 10.13 \pm 0.08$) than of noradrenaline at β_1 adrenergic receptors ($-\log K_B = 9.02 \pm 0.07$). On the other hand, propranolol is a nonselective agent with equal affinity for β_1 and β_2 receptors (Reiter, 2004). Moreover, in the final stage of heart failure, carvedilol treatment reduced 1.8-

fold and 25.1-fold the sensitivity of right ventricular trabeculae to noradrenaline and adrenaline, respectively (Molenaar *et al*., 2005). This result indicates that carvedilol may reduce overactivity of catecholamine.

In the present study, the beta 2 adrenergic receptor density was not changed by L-thyroxine administration when compared with the control group, and this change differs from several studies. Several studies have shown that thyroid hormone treatments have been found to increase receptor densities in animals (Banerjee and Kung, 1977; Tse *et al*., 1980; William *et al*., 1977). However, there is a study which shows no changes in receptor numbers, influenced by thyroid hormones (Stiles and Lefkowitz, 1981). According to the results of our study, it is possible that the chronic effects of thyroid hormones on adrenergic receptor numbers that occur in clinical hyperthyroidism are different from the effects of acute changes in thyroid hormone levels induced by experimental hyperthyroidism, or may be explained by the masking effect of inter-individual variability.

Therefore, the mechanism of a decrease in factor VIII levels caused by carvedilol but not propranolol may be due to the persistently greater blockade of β_2- than β_1 adrenergic receptors and property of the stabilizing effect. However, finding on changes of beta adrenergic receptors of mononuclear leukocytes in cats during L-thyroxine administration failed to give a clear answer. This issue needs further investigations.

Previous studies have shown that the rise of vasopressin associated with hyperthyroidism lead to increases in factor VIII levels, but we did not investigate these actions. Thus, future research assessing the relationships between increases in vasopressin and hyperthyroidism may be required. Furthermore, measurements of catecholamine levels may improve result interpretation of factor VIII levels regarding the action of catecholamines on factor VIII levels.

In conclusion, the present study suggests that carvedilol can decrease factor VIII levels. This action may provide an important information on prevention of thromboembolism in hyperthyroid cats. The principle of reduction mechanism is may be due to a decrease in thyroxine level that leads to a reduction of protein synthesis, including factor VIII. Another mechanism may be due to an inhibition of factor VIII release from endothelial cells via the β_2-adrenergic receptor pathway.

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APPENDIX

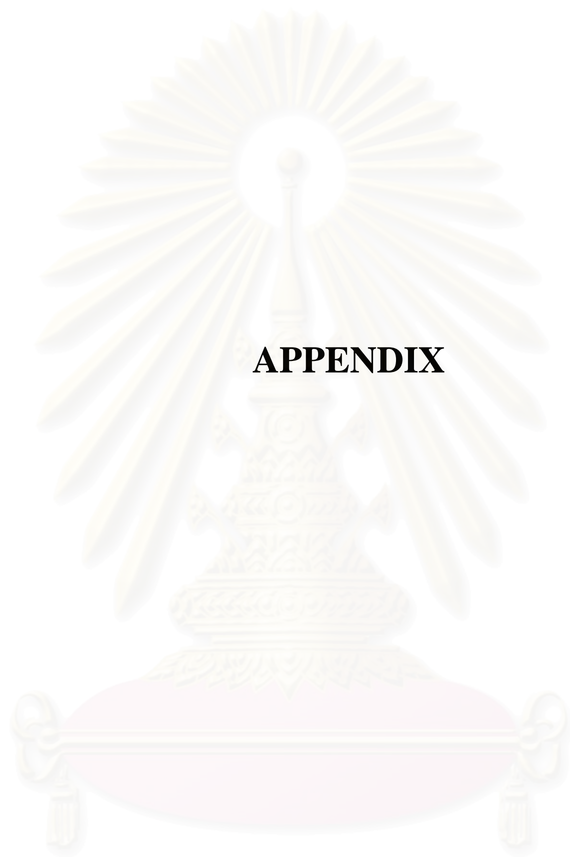

Table i Hematology and blood chemistry profile of cats in the control group.

Numbers / Hemato/Chem	1 (female)	2 (male)	3 (male)	4 (male)
R.B.C. ($x10^6/\mu$)	10.06	11.3	7.23	11.06
Hemoglobin (g/dl)	15.8	18.7	12.4	17.6
Hematocrit (%)	50.4	56.4	38.8	54.8
MCV (fl)	50.1	49.8	53.6	49.6
MCH (pg)	15.7	16.5	17.0	15.9
MCHC (%)	31.4	33.2	31.9	32.1
W.B.C. (/µ)	20,000	8,900	17,500	17,600
Band-N (%)	-	1	-	-
Seg-N (%)	75	85	61	59
Lymphocyte (%)	21	11	36	30
Monocyte (%)	-	-	-	-
Eosinophil (%)	4	3	3	11
Basophil (%)	-	-	-	-
N-Myelocyte (%)	-	-	-	-
N-Metamyelocyte (%)	-	-	-	-
NRBC (100/WBC)	-	-	-	-
Platelet (/µl)	231,000	297,000	89,000	115,000
Blood parasite	NF	NF	NF	NF
RBC Morphology	Normal	Normal	Normal	Normal
SGPT (IU/L)	56.0	124.2	70.1	144.5
Creatinine (mg/dl)	1.3	1.2	1.3	1.7

All cats were FIV, FeLV Negative.

Table ii Hematology and blood chemistry profile of cats in the hyperthyroid group.

Hemato/Chem \ Name	1 (female)	2 (male)	3 (male)	4 (female)	5 (male)	6 (male)	7 (female)	8 (female)
R.B.C. ($x10^6/\mu$)	8.13	9.52	9.23	7.05	8.77	7.36	9.09	8.13
Hemoglobin (g/dl)	12.4	13.9	14.5	10.6	12.9	13.0	13.6	12.4
Hematocrit (%)	37.6	44.3	43.9	32.8	40.4	43.5	42.9	37.6
MCV (fl)	46.2	46.5	47.6	46.6	46.1	59.1	47.1	46.2
MCH (pg)	15.3	14.6	15.7	15.0	14.8	17.6	15.0	15.3
MCHC (%)	33.0	31.5	33.0	37.2	32.0	29.8	31.8	33.0
W.B.C. (/µ)	23,700	17,800	10,900	14,800	26,900	22,400	16,700	23,700
Band-N (%)	1	-	-	-	-	-	-	1
Seg-N (%)	68	62	79	84	81	60	67	68
Lymphocyte (%)	29	26	21	12	16	37	28	29
Monocyte (%)	1	-	-	4	-	-	-	1
Eosinophil (%)	1	12	-	-	3	3	5	1
Basophil (%)	-	-	-	-	-	-	-	-
N-Myelocyte (%)	-	-	-	-	-	-	-	-
N-Metamyelocyte (%)	-	-	-	-	-	-	-	-
NRBC (100/WBC)	-	-	-	-	-	-	-	-
Platelet (/µl)	223,000	18,700	181,000	170,000	212,000	80,000	266,000	223,000
Blood parasite	NF	NF	NF	NF	NF	NF	NF	NF
RBC Morphology	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
SGPT (IU/L)	85.6	72.0	46.6	58.3	104.0	50.3	139.8	85.6
Creatinine (mg/dl)	1.1	1.0	1.0	1.2	1.3	1.4	0.8	1.1

All cats were FIV, FeLV Negative.

Table iii Body weight (kg) of cats in the control group during baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	2.18	2.25	2.15
2	4.00	4.00	4.00
3	3.75	3.70	3.70
4	2.15	2.20	2.10
Means	3.02	3.04	2.99
SEM	0.50	0.47	0.50

Table iv Body weight (kg) of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	2.32	2.30	2.15	4.45
2	4.24	4.33	4.35	4.45
3	3.41	3.53	3.45	3.65
4	3.50	3.35	3.20	3.25
5	4.91	4.85	4.90	4.90
6	2.82	2.95	3.00	3.10
7	2.44	2.30	2.25	2.25
8	4.12	4.01	3.95	3.50
Means	3.47	3.45	3.41	3.69
SEM	0.33	0.33	0.34	0.31

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Table v Thyroxine levels (nmol/L) of cats in the control group during the baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	23.17	18.02	18.02
2	17.37	14.16	16.73
3	12.99	8.49	11.84
4	25.10	30.88	32.18
Means	19.66	17.89	19.69
SEM	2.76	4.75	4.37

Table vi Thyroxine levels (nmol/L) of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	25.68	66.86	15.32	20.85
2	19.63	82.75	18.66	55.86
3	22.97	38.42	37.07	31.53
4	21.04	62.16	25.87	8.37
5	10.17	96.46	69.50	27.03
6	15.77	88.61	23.17	66.92
7	23.55	108.37	46.33	100.39
8	29.34	90.93	14.16	46.33
Means	21.02	79.32	31.26	44.66
SEM	2.11	7.90	6.71	10.43

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Table vii PT values (sec) of cats in the control group during the baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	14.1	15.8	14.1
2	15.0	15.9	13.9
3	16.0	15.7	15.0
4	12.9	12.5	12.6
Means	14.46	15.0	13.9
SEM	0.66	0.83	0.49

Table viii PT values (sec) of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	13.3	14.4	14.1	13.7
2	12.4	12.9	12.3	13.0
3	NA	NA	NA	NA
4	NA	NA	NA	NA
5	12.4	12.9	12.8	13.4
6	13.2	12.7	12.9	12.2
7	13.6	12.9	14.1	13.1
8	13.5	13.3	12.2	13.2
Means	13.04	13.16	13.07	13.10
SEM	0.21	0.25	0.35	0.21

NA = Not available due to clot formation of sample before test.

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Table ix APTT values (sec) of cats in the control group during baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	13.7	14.2	12.6
2	11.8	18.0	13.7
3	10.3	12.3	11.5
4	9.1	8.4	6.4
Means	11.23	13.23	11.05
SEM	0.99	2.00	1.61

Table x APTT values (sec) of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	21.0	18.2	22.1	26.4
2	19.0	17.8	15.9	11.7
3	NA	NA	NA	NA
4	NA	NA	NA	NA
5	21.7	15.0	10.7	17.3
6	13.5	13.1	15.8	8.9
7	18.0	15.3	7.9	22.3
8	10.0	7.5	9.8	7.9
Means	17.17	14.5	13.7	15.8
SEM	1.86	1.59	2.14	3.08

NA = Not available due to clot formation of sample before test.

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Table xi Factor VIII levels (IU/ml) of cats in the control group during baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	7.79	6.90	8.73
2	5.63	5.92	6.59
3	6.75	7.91	8.86
4	4.59	5.10	1.66
Means	6.19	6.46	6.46
SEM	0.69	0.61	1.68

Table xii Factor VIII levels (IU/ml) of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	4.88	8.35	5.92	6.63
2	6.15	6.55	5.39	5.81
3	4.59	7.16	6.06	5.77
4	5.75	14.04	14.13	11.65
5	6.84	8.02	9.09	7.79
6	12.47	12.64	11.23	10.87
7	6.96	8.52	9.50	8.90
8	10.41	12.35	10.20	9.72
Means	7.26	9.70	8.94	8.39
SEM	0.98	1.01	1.07	0.80

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Table xiii β 2 adrenergic receptor densities of cats in the control group during the baseline, and beta adrenergic antagonists administrations.

No	Baseline	Propranolol	Carvedilol
1	0.855	0.044	1.000
2	0.745	1.187	0.209
3	1.092	0.896	0.812
4	0.251	0.599	0.091
Means	0.736	0.682	0.528
SEM	0.18	0.24	0.22

Table xiv β 2 adrenergic receptor densities of cats during L-thyroxine, L-thyroxine combination with propranolol, and L-thyroxine combination with carvedilol administrations.

No	Baseline	Thyroxine	Propranolol	Carvedilol
1	0.323	0.405	0.413	0.281
2	0.429	0.338	0.311	0.305
3	0.650	0.714	1.093	0.413
4	0.491	0.602	0.498	0.278
Means	0.473	0.514	0.579	0.319
SEM	0.07	0.09	0.18	0.03

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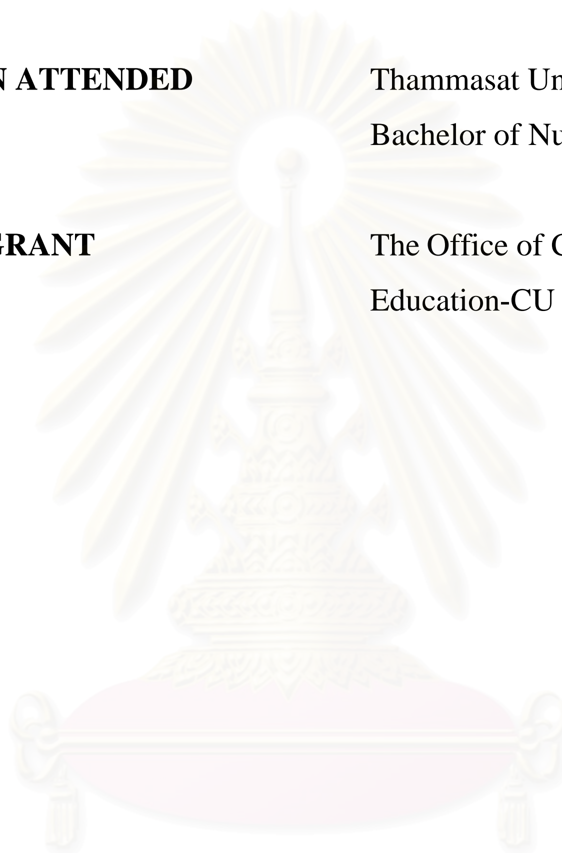

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