CHAPTER II

BACKGROUND INFORMATION

1. The role of maternal thyroid hormones

The thyroid function in pregnancy is important because clinically abnormal thyroid function interferes with initiation of pregnancy, maintenance of pregnancy, and the normal outcome of pregnancy (Vanmiddlesworth, 1981).

Cretin, characterizing by multiple neurological defects, is the clinical outcome of maternal thyroid hormone deficiency (Pharoah *et al.*, 1971; 1972; 1980; Pharoah and Hornabrook, 1974; Delong *et al.*, 1985; Delong 1987; Chaouki *et al.*, 1988). The endemic cretinism can be prevented by a given sufficient iodine to the mother prior to conception (Pharoah *et al.*, 1971; 1972). The damage to the fetus appears to occur early in pregnancy, probably during the first trimester (Pharoah *et al.*, 1980). In a follow-up study of pregnant women in an area of severe iodine deficiency in Papua New Guinea, more stillbirth infant deaths and endemic cretinism occur among the offspring of women suffering from severe iodine deficiency (Pharoah *et al.*, 1976). It is of interesting that the deficiency produces abnormally low serum total and free thyroxine (T4, fT4) without clinical evidences of hypothyroidism (Greer *et al.*, 1968; Delange *et al.*, 1972);

euthyroidism is maintained by a normal or possibly a raised serum tri-iodothyronine (T3) levels (Pharoah *et al.*, 1973; Chopra *et al.*, 1975). Thus, in areas of dietary iodine deficiency, the observed range of serum T4 is wide with significant proportion of the population having T4 and fT4 values below the normal range (Pharoah *et al.*, 1972). In 1981, Pharoah and his colleagues studied the relationship between materal thyroid hormones levels and the motor performance (as a reflection of neurological integrity) of her offspring in childhood and reported a significant correlation between the children's motor performance and maternal serum T4 levels, but not with T3 level.

It is widely accepted that thyroid hormones play an important role in the central nervous system (CNS) development and the most striking effects could be seen during the period of CNS maturation (Hetzel and Potter, 1983; Dussault and Ruel, 1987). The absence of thyroid hormones during CNS maturation can produces multiple morphological and biochemical alterations which leads to irreversible mental retardation in man (Dassault and Ruel, 1987; Delong, 1987), monkeys (Kerr *et al.*, 1972; Bachrach *et al.*, 1983; Mano *et al.*, 1985; Mano *et al.*, 1987), rats (Balazs *et al.*, 1969; Takahashi *et al.*, 1981; Chaudhury and Sarkar, 1983, Morreale De Escobar *et al.*, 1985; Escobar Del Rey *et al.*,1986; 1987; Mooradian, 1990), sheeps (McIntosh *et al.*, 1983; 1986; 1987; Potter *et al.*,

1986; Mano *et al.*, 1989). Recent convincing data are now available demonstrating an important effects of maternal thyroid hormones on the foetal development during the early stage of pregnancy. The idea of the fetal requirement for T4 is implicited in a hypothesis advanced by Ekins (1984 a; 1984 b;1985;1989) in which the rise in maternal thyroxine binding globulin (TBG) in human pregnancy is regard as fascilitating T4 delivery to the fetoplacental unit. Contrary to the traditional understanding, there is no transport of T3 or T4 in late pregnancy in sheeps (Hopkins and Thorburn, 1971) and humans (Dussault *et al.*, 1972) eventhough others suggest that in rabbit (Osorio and Myant, 1960) and human (Grumbach and Werner, 1956; Myant, 1958) conditions become more favourable during mid to late pregnancy. Early work on the uptake of T3 and T4 by the rat fetus suggested minimal transport of T4 (Dussault and Coulombe, 1980) and no transport of T3 (Dubois *et al.*, 1977). However, the transfer of maternal thyroid hormones from the mother to the rat fetus early in gestaion has been shown with different methodologies (Obregon *et al.*, 1984 a ;1984 b; Woods *et al.*, 1984; Morreale de Escobar *et al.*, 1985; Ekins *et al.*, 1989). These reports claim that maternal hypothyroidism is accompanied by fetal thyroid hormone deficiency before the onset of independent fetal thyroid function (17 day gestation). Furthermore, thyroid hormones were undetectable in embryos and placentas

obtained before the onset of fetal thyroid secretion following maternal thyroidectomy. Recent report of Obregon *et al*(1984a) in rat embryonic tissues are provided with T4 and T3 only 4 days after uterine implantation and well before the onset of fetal thyroid function at 17 day gestation. Moreover, thyroid hormones and their nuclear receptors have been found in rat and human fetuses before the thyroid gland become active (Bernal and Pekonen, 1984). The simultaneous presence of thyroid hormones and their receptors indicate a biological effects early in gestation.

2. The significance of thyroxine-binding globulin (TBG) related to free and total thyroxine (fT4, T4)

It is commonly believed that thyroid hormone binding proteins assist in retention of thyroid hormones within the vascular compartment preventing glomerular loss, flooding into cells and transplacental passage from mother to fetus (Osorio and Myant, 1960). These binding proteins also regard as hormone reservoirs which attenuate fluctuations in serum hormone levels bring about by short term alterations in secretion or peripheral demand (Ingbar, 1985). These conventional ideas do not explain the large inter-species differences and greater thyroid binding protein concentrations during pregnancy (Ekins, 1984 a; 1985; 1988; 1989 a ; 1989 b ;Ekins *et al.*, 1989). The extent and significance of maternal thyroid hormones transport to the fetus has recently been discussed and it

has been pointed out that T4 rather than T3 is needed for brain development. Accordingly, correlations between maternal hypothyroidism and intellectual deficit in the offspring also point to a possible T4 requirement by the feto-placental unit (Ekins, 1982;1985; Ekins and Edwards, 1988). Moreover, the hypothesis is purposed and embraced the ideas that

(a) there exists a specific feto-placental requirement for T4 throughout pregnancy,

(b) TBG and other serum thyroid hormone binding proteins exist for ensuring adequacy of T4 supply to the feto-placental unit,

(c) T4 is conveyed to the fetus early in pregnancy prior to the development of the fetal thyroid gland,

(d) T4 by its de-iodination in the placenta in later pregnancy serves as a principal source of iodine to the fetus, particularly in circumstance of dietary iodine deficiency when maternal serum iodide levels are likely to be low (Ekins *et al.*, 1982; 1989; Ekins, 1984).

However, the original concept purpose that both T4 and T3 express their physiological activities only when they are in the free form (Ekins, 1978; 1979;1981;1987;1990; Pardridge, 1987; Mendel, 1989). The concentration of fT4 in serum correlates more closely with clinical thyroid status than the levels of T4 or T3 (Bayer and McDougall, 1980). In order to exert at the cellular

functions, a number of related conclusion are (1) T4 enters target cells and detaches from serum transport proteins (2) its rate of entry (and hence its physiological effect) is governed by the serum 'free' hormone concentration as established in a hormone/serum protein mixture in thermodynamic equilibrium; and (3) its rate of entry into pituitary thyrotrophic cells (and hence its modulating effect on the hypothalamic-pituitary-thyroid feedback system) is likewise governed by the 'free' hormone concentration (Ekins, 1978; 1981; 1982 a; 1982 b ; 1984 a ; 1990). The relationship between total and free thyroxine concentrations and the levels of the thyroid hormone binding protein in serum are generally accepted as being governed by the law of mass action (Ekins, 1978; 1981; 1982 a ; 1982 b ; 1984 a ; 1990; Woeber, 1987). Therefore, the proportion of free hormone will be a major determinant of the distribution and fractional turnover rate of hormone and varies inversely with the binding affinity and concentration of unoccupied protein-binding sites (Ingbar and Freinkel, 1960; Woeber, 1987). The concentration of free hormone will be a major determinant of the absolute rate of hormone flux into the cell to exert intracellular metabolism and depend on the total concentration of hormone in the plasma (Woeber, 1987). Moreover, the perturbations of the thyroid hormone binding protein interaction may result primarily from alterations in hormone binding, principally the

concentration of thyroxine binding globulin (TBG) or from changes in hormone supply (Woeber, 1987). The most common natural cause of increased TBG is pregnancy recently reports in women (Laurell and Ramevik, 1979, Skjoldebrand *et al.*, 1982; 1987; 1988, Price *et al.*, 1989) and in cynomolgus monkeys (Suwanprasert *et al.*, 1989; 1990). There are different patterns of binding capacities of thyroxine binding proteins in various species of vertebrates (Tanabe *et al.*, 1969, Refetoff *et al.*, 1970; Larsson *et al.*, 1985; Ando *et al.*, 1986). No apparent TBG was detected in laboratory rodents (Tanabe *et al.*, 1969, Larsson *et al.*, 1985). But the presence of TBG was demonstrated in larger mammals (Tanabe *et al.*, 1969, Refetoff *et al.*, 1970, Larsson *et al.*, 1985) including monkeys (Glinoer *et al.*, 1977a; 1977b; 1979, Mcguire *et al.*, 1982, Ando *et al.*, 1986). An increased TBG and steroid sensitive proteins of pregnancy in women are due to enhanced secretion of endogenous estrogens from the feto-placental unit which in turn stimulate increments of hepatic TBG synthesis (Laurell and Rannevik, 1974, Woeber, 1987). Estrogens in oral contraceptive agent also induce serum elevation of TBG, T3 and T4 levels (Song *et al.*, 1970; Barbosa *et al.*, 1973). Although highly significant correlation between TBG and estrogen levels are obtained throughout entire pregnancy in women, the correlation within two-week intervals of pregnancy are rather poor and mostly insignificant (Skjoldebrand *et al.*, 1987). Nevertheless, estrogens may have a modulatory function in

'steroid sensitive protein' synthesis, but may not be the main physiological regulator (Skjoldebrand, 1988). However, the fT4 concentrations have been reported to decrease slightly or increase during pregnancy in spite of elevation of both T4 and TBG (Ekins 1978, Boss and Kingstone, 1979; 1981; Price *et al.*, 1989). Obviously, controversial results are due to the different methods for measuring unbound thyroid hormone as analog (one step) or non-analog (two-step) determination which base on different understanding concepts in free hormone hypothesis (Ekins,1979; 1983; 1984 a ; 1987; 1989 a ;1989 b ; 1990 ; Ellis and Ekins, 1975, Symon *et al.*, 1983; Wilke, 1982; 1986; Liewendahl *et al.*, 1984; Wilkins, 1987; Mahadevan and Ooi, 1988; Wirquin *et al.*, 1987; Mendel, 1990).

The mechanism of estrogen action on stimulation of TBG synthesis in the liver were further studied in the rhesus monkey and described briefly. (Glinoer *et al.*, 1976; 1977 a ; 1977 b ; 1979; Mcguire *et al.*, 1982). The liver of young adult monkeys was perfused *in situ* through the portal vein with collagenase, hyaluronidase and EDTA (Glinoer *et al.*, 1977 b ; Robbins *et al.*, 1978). About 50-70 million cells per gram of liver, of which more than 90% were well preserved prior to incubated with ^{3}H-or ^{14}C-leucine. Only a very small amount of total radioactive amino acid incorporated into protein and then was recovered in TBG- like protein fraction . Rapid

production of TBG showed about 20 times greater than normal in only 18 hours. Hepatocytes previously exposed to B-estradiol (E_2) were capable of releasing TBG at a much faster rate (2.4 times normal). Sawhney and his colleagues (1978) reported the prolonged estradiol monobenzoate treatment reduced the metabolic clearance rate (MCR), distribution space and production rate (PR) of both T3 and T4. Injection of highly purified rhesus monkey TBG labeled with ^{125}I to the monkeys, the radiolabeled TBG disappeared from blood with a half-life of 2.7 $\pm$ 0.1 days and showed prolonged half-life when treatment with E_2 before. The most striking effect of estrogen was 2.75-fold increase in TBG production, mainly brought about by a change in the synthesis and secretion of TBG by the liver (Glinoer *et al.*, 1977a; 1977b). During the control period, TBG was distributed as following : serum equivalent volume (SEV), 323 $\pm$ 23 ml/3 kg BW; the final decay rate (k), 0.28 $\pm$ 0.01 day; the metabolic clearance rate (MCR), 92 $\pm$ 10 ml/day and the production rate (PR), 2.23 $\pm$ 0.14 mg/day (Glinoer *et al.*, 1979). In the hypothyroid state, opposite changes were observed in serum TBG (21% increase); k (51% decrease); SEV (17% increase), but not in MCR (43% decrease) or PR (27% decrease) (Glinoer *et al.*, 1979). Since TBG is primarily T4-rather than T3-binding protein, as evidenced by its higher affinity for T4 and the much higher levels of T4-TBG complex in plasma (Robbins *et al.*, 1978).

Only T4 can therefore elevate either MCR or PR of TBG to normal in thyroidectomized monkeys but both T3 and T4 replacement restored serum TBG and K to euthyroid levels (Mcguire *et al.*, 1982). In man, elevated TBG capacity with markedly diminished fT4 fractions is mostly found but normal both TBG capacity and fT4 fraction is also reported (Inada and Sterling, 1967).

3. Hypothyroidism and ovarian function

Although the hypothyroidism may occurs throughout the entire life span, the peak incidence tends to occur after the childbearing period during the age range of 30-60 years. Thus, hypothyroidism is relatively uncommon in the pregnant women (Burrow, 1980).

3.1 Effects of ovarian steroids upon thyroid status

It has long been recognized that estrogenic steroids are capable of stimulating the TBG production in man (Song *et al.*, 1970; Barbasa *et al.*, 1973; Skjoldebrand *et al.*, 1987; 1988) and old world monkeys (Glinoer., 1977 a ; 1977 b ; 1979;Sawhney *et al.*,1978; Mcgurire *et al.*,1982). The effect definitely follows by transient decrease in serum fT4 levels in compensate for increased endogenous T4 production. This result is sufficient to rise total plasma T4 pool and normalize the fT4 level. The response of TBG to estrogen is dose-related (Doe *et al.*, 1967; Robbin *et al.*, 1978; Sawhney *et al.*, 1978;

Zaninovich, 1981) On the other hand, progestin component in oral contraceptives shows no apparent effects upon serum levels of thyroid hormones (Winikoff, 1968). During the normal menstrual cycle, small fluctuations in thyroid hormones levels are detected both in women (Beck *et al.*, 1972; Weeke and Hansen, 1975; Hegedus *et al.*, 1986) and in cynomolgus monkeys (Suwanprasert *et al.*, 1987). Recently, serum TSH and thyroid volume have been exhibited a similar increase with a positive correlation between serum thyroglobulin and thyroid volume on day 2, 9, 16, 23 and day 2 of next cycle in women (Rasmussen *et al.*, 1989). Evidence shows the responsiveness of TSH to TRH is greater during the preovulatory phase than the luteal phase of normal menstrual cycle (Sanchez - Franco *et al.*, 1973), although no significant changes in TSH levels detect throughout the cycle (Fukuda, 1975). In man, treatment with ethinyl estradiol affects basal TSH levels without a significant alteration of circulating T4 (McNeilly, 1986). An enhanced response of both TSH and PRL to TRH has been reported in the women taking oral contraceptive agent (Ramay *et al.*, 1975). In adult female rats, daily 2.0 ug estradiol benzoate (E_2B) injection for 7 days suppressed serum T4 levels but elevated T3 levels with a greater TSH response to TRH (Chen and Walfish, 1978). Prolonged treatment of E_2B resulted decrease in MCR and PR of both T3 and T4 in rhesus monkeys (Sawhney *et al.*, 1978). In women, the effect of estrogen upon the

peripheral metabolism of T4 showed a decrease in the fractional T4 turnover rate (Dowling *et al.*, 1960), hence, in the rate of hormone clearance from its peripheral distribution space. Concomitantly, both the concentration and total quantity of hormone within the space increased so that the absolute rate of hormonal disposal remained unchange. Additional evidence reported by Beierwaltes and Robbins (1959) in 2 types abnormal TBG, idiopathic increase in TBG and the other was virtually devoid of TBG. The first type of patients suffering from idiopathic greater TBG showed decrease in turnover of T4 whereas the second type of patients presented unaltered kinetics of T4 metabolism after administration of estrogen. The data to date indicate that decrease in metabolic degradation of T4 and increase in T3 which both result from an increase in the binding activity of TBG (Robbin *et al.*, 1978). Pharmacological dose of E_2 increases the PBI as well as serum T4 concentrations by one and one-half to two times, and this effect is secondary to a two-to three-fold increase in serum concentration of TBG and similar to the case found in taking estrogen contraceptive pills (Gregerman, 1985). On the other hand, hypothyroidism would resulted in increment of both MCR of testosterone and conversion ratio of testosterone to androstenedione (Gordon *et al.*, 1969). It is noted that the conversion ratio of androstenedione to estradiol increased only in male but not in the female (Southern *et al.*, 1974). Apart from these, estrogens are capable of influencing upon

thyroidal accummulation of released iodide. Strong evidence for estrogen accelerated thyroid radioiodide release in human infants (Fisher and Oddie, 1963) but the accumulation of radioiodide and hormone release rate are suppressed by estrogen in adult (Gross *et al.*, 1975).

3.2 Influences of hypothyroidism on patterns of menstrual bleeding.

Hypothyroidism has been implicated as the underlying cause of many irregularities of menstruation. Bleeding may be excessive, acyclic or diminished (Hodges *et al.*, 1952; Scott and Mussey, 1964). Menorrhagia is observed in myxedematous patients with hyperplastic proliferative changes in the uterine endometrium and unopposed estrogen secretion (Goldsmith, 1952). These may possibly be due to either a defect in uterine muscle contraction (Ross *et al.*, 1958) or a direct effect of deficient thyroid hormone on the endometrial response to estrogen (Longcope, 1985).

3.3 Influences of hypothyroidism on ovarian steroid production.

The incidence of hypothyroidism is greater in female than in male and seems to affect obviously with the ovarian endocrinology. Many convincing data are presented and based on animals as well as human studies.

3.3.1 Animal studies

In 1941, Williams and his coworkers firstly observed severe abnormalities of the cycle associated with 10% reduction in BMR following thyroidectoncy in guinea pig. Prolonged thiouracil adminstration (24 mg/day/rat) in the adult female rat will terminate pregnancy (Seegar-Jones *et al.*, 1946). Hypothyroid state tends to result in a depressing of pituitary gonadotrophic activities (Hagino, 1971). In thyroidectomized rats, the age of vaginal opening is not significantly altered but the average time of vaginal cornification is slightly prolonged. However, ovulation cannot detect on the day of vaginal opening as occurs normally. Saiduddin (1972) reported that hypothyroidism lessened ovarian weight but unable to prevent compensatory ovarian hypertrophy in unilateral ovariectomized rats. Jones and Tracy (1974) further suggested that hypothyroidism in rats may suppress normal pattern of pituitary gonadotrophin secretion. In hypothyroid sheep, the uterus shows endometrial hyperplasia and mesometrial hypertrophy associate with prolonged estrus appearance (Nesbitt *et al.*, 1967).

Much of these earlier works were carried out prior to the establishment of sensitive technique RIA, and therefore, the degree of hypothyroidism could not accurately correlated with the associated disturbances in reproductive function. Extensive studies on relationship between hypothyroid state and reproduction are further

reported by Larochelle and Freeman (1974) that serum levels of LH and FSH of athyroid-ovariectomized rats increased 50-180% higher than those found in euthyroid-ovariectomized animals but decreased to the control levels after daily administration of T4 in the dose of 5 ug/100 gm BW/day, although MCR of both LH and FSH did not alter in both groups. They suggested that thyroid hormones play a role in modulating the secretion of both LH and FSH and this effect was not due to alteration of the MCR of gonadotrophins. On the other hand, physiological or supra-physiological doses of T4 suppressed serum levels of LH which similar to those found in euthyroid-ovariectomized rats (Freeman *et al.*, 1975). In long-term ovariectomized rats sampled at 5-10 min intervals, plasma LH concentration was not elevated markedly but fluctuated with a periodicity of 15-30 min (Gay and Sneth, 1972). In view of the fact that thyroid hormone changes the secretion rate of LH in ovariectomized rats and may be a reflection of change in pulsatile pattern of gonadotrophin secretion. The frequency of the plasma LH rhythm in untreated athyroid-ovariectomized rats was normal but the maximum and minimum concentrations were 2-to 3-fold higher than those of euthyroid-ovariectomized rats. However, treatment of athyroid-ovariectomized rats with a daily dose of T4 (20 ug/100 gm BW) for 8 days, attenuated pulsatile discharges of LH. Furthermore, evidences suggested that altered thyroid status did not influence

the synthesis and metabolism of LH but did exert a profound effect on the secretion of this hormone, presumably by acting directly on the hypothalamo-pituitary axis (Freeman *et al.*, 1975). The effects of thyroid status on the preovulatory surge of LH was further investigated in the ovariectomized-thyroidectomized (OV-TX) rats maintained on a physiological regimen of T4 (2 ug/100 g BW/day) comparing with OV-TX rats without T4 replacements indicated that larger dose of estrogen is needed to induce an LH surge (Freeman *et al.*, 1976). Recently, Maruo *et al*(1987) demonstrated direct effect of T3 and T4 on porcine granulosa cells *in vitro* and found that combine treatment with FSH and T4 (10^{-7}M) induced morphological alteration of epitheloid cells, while either FSH or T4 alone failed to bring about the epitheloid morphology. Concomitant treatment with FSH and T4 (10^{-7}M) markedly increased FSH stimulated induction of [^{125}I] iodo-human CG binding to cultured granulosa cells obtained from small follicles. The combined treatment with FSH and T4 (10^{-7}M) also resulted in a significant increase in progesterone and estrogen secretion from the cultured cells relative to treatment with FSH alone. An increase in progesterone (P), 17B-estradiol (E_2) and estrone (E_1) secretion caused by the combined treatment with FSH and T4 (10^{-7}M) were further augmented by addition of these steroids substrates,including pregnenolone, testosterone and androstenedione.Furthermore, aromatase activity assessed by the release of [^{3}H] water from [1B-^{3}H-4-^{14}C]

androstenedione was significantly higher in cells treated concomitantly with FSH and T4 (10^{-7}M) than that in cells treated with FSH alone. Similar-responses were also obtained if the cultured granulosa cells exposed with FSH and T3 (10^{-9}M). However, either higher of lower doses or T3 of T4 gave attenuated effects, whereas T3 of T4 alone without FSH was incapable of exhibiting these stimulatory effects. They suggested that both T3 and T4 were able to synergize with FSH to exert direct stimulatory effects on granulosa cell function, morphological differentiation, LH/hCG receptor formation and steroidogenic enzyme activation (3B-hydroxy dehydrogenase and aromatase). Hence, decrease in ovarian functions during the states of hypo-or hyperthyroidism may account for diminished responsiveness of the granulosa cells to FSH. Interestingly, Bhattacharya *et al.*, (1989) found a high affinity of T3 receptors in highly purified nuclear preparations from human corpus luteal and from ovarian tissue of freshwater perch, *Anabas testudineus*. The functional relevance of T3 receptor in the ovary was studied by using perch oocytes homogenated and separated 150 K supernatant by ultracentrifugation. The results showed that T3 induced protein synthesis in the oocytes and when 150 K supernatant from T3 treated oocytes was added to mitochondrial incubation, formation of pregnenolone from cholesterol was greatly increased. Addition of T3 antisera blocked the stimulatory effect of

150 K supernatant. When 150 K supernatant from T3 incubated oocytes was filtered through sephadex G-100 column, a new protein peak was obtained. This clearly indicated that T3 induced the synthesis of a new protein, the molecular weight of which was less than 10,000 dalton. Furthermore this T3 induced protein (T3P) incubated with oocytes *in vitro* release large amount of progesterone. The findings indicated that binding of T3 with ovarian receptors induced the generation of a low molecular weight protein which in turn stimulated progesterone formation and release (Bhattacharya *et al.*, 1989).

3.3.2 Human studies.

The earliest and most common abnormalities observed in hypothyroid women were changes in uterine bleeding and in the length of the intermenstrual intervals (Roger, 1958). In prolonged myxedema, amenorrhea is often present, alternating with periods of abnormal uterine bleeding of varying severity. The cause of the infertility in hypothyroid women is two folds. First, amenorrhea, galactorrhea and hyperprolactinemia are commonly found in myxedematous women (Edwards *et al.*, 1971; Boroditsky and Faiman, 1973; Fish and Mariash, 1988; Tkachenko *et al.*, 1989). Second, pregnancy is unusual in hypothyroidism suggesting fertility and thyroid function are closely interrelated (Burrow, 1980; Hembrec and Wiele, 1986). Nonetheless, patients with untreated hypothyroidism have been reported to carry successfully

pregnancies to term (Hodges *et al.*, 1952). Since maternal thyroid hormone would be lacking, the fetus would not be exposed to any significant thyroid hormone during the first trimester (Pharoah *et al.*, 1971; 1976; 1981; Ekins, 1985; Ekins *et al.*, 1989). The occurrence of congenital defects including mental retardation, mongolism and clawfoot deformity have been reported in the children from the hypothyroid mothers (Hodges *et al.*, 1952). Moreover, down's syndrome and ostium primum defect are also found in the infant delivered from hypothyroid mothers (Montoro *et al.*, 1981). As mentioned, mild hypothyroidism is compatible with ovulation and conception, but when pregnancy occur, abortion; prematurity or stillbirth is very common (Greenman *et al.*, 1962; Montoro *et al.*, 1981). In more severe disease, anovulation is the usual cause of failure to conceive. The cessation of irregular menstruation and anovulation is rapid and dramatic after institution of levothyroxine replacement. An ovulatory cycle may be evidenced within 1-2 months after treatment and pregnancy could be achieved in many cases (Fish and Mariah, 1988). Hypothyroidism in prepubertal girl may associate with delay in growth, thelarche and menarche (Ingbar, 1985). However, an interesting syndromes described by Van Wyk and Grumbach (1960) are precocious menstruation and galactorrhea in girls with juvenile hypothyroidism. It was thought that these syndromes are result of an overlap in the pituitary production of TSH

and gonadotrophin during the early ovarian stimulation. Higher estrogen production which in turn stimulate early endometrial changes and vaginal bleeding is purposed (Goldsmith *et al.*, 1952; Ross *et al.*, 1958; Van Wyk and Grumbach, 1960). Lower thyroid hormones stimulate the TSH release and turn toward enhance TRH secretion (Kourides *et al.*, 1984; Gregerman, 1986). Subsequently, TRH augments the levels of PRL increase and which may lead to galactorrhea (Edwards *et al.*, 1971; Snyder *et al.*, 1973; Toft *et al.*, 1973; Boroditsky and Faiman, 1973; Gershengorn, 1985; Longcope, 1986). However, adrenal production of androgen precussors is still fairly lowered so that auxillary and pubic hairs are usually not apparent (Van Wyk and Grumbach, 1960, Longcope, 1986). With regard to the response of gonadotrophin, Distiller and his colleagues (1975) reported in eight hypothyroid women that gonadotrophin response bluntly to gonadotrophin-releasing hormone (GnRH) (100 ug) . Results of GnRH test indicated that there is limited pituitary LH reserve in primary hypothyroidism patients. This finding is supported by low T4 syndrome in critically ill postmenopausal women with hypogonadotropism (Quint and Kaiser, 1985). Recently, Tkachenko and his coworkers (1989) reported that hyperprolactinemia developed in cases of primary hypothyroidism is associated with impaired dopaminergic inhibition of pituitary lactotrophs.

4. Hyperprolactinemia : common complication during hypothyroidism.

Hyperprolactinemia is the condition of which there are excessive levels of prolactin (PRL) in the circulation a level higher than 800 mIU/L in man (Lenton *et al.*, 1979) and over 1,500 mIU/L in cynomolgus monkeys (Varavudhi *et al.*, 1990), one is found in association with lactation (Gautvik *et al.*, 1973; Noel *et al.*; 1974; Schallenberger *et al.*, 1981; Aso and Williams, 1985) while another occurs during primary hypothyroidism (Ross and Nusynowitz, 1968; Edwards *et al.*, 1971; Boroditsky and Faiman, 1973), pituitary tumor (Kleinberg *et al.*, 1977; Frantz, 1978; Chang, 1983; Christopoulos, 1986; Fish and Mariash, 1988) and drug-induced hyperprolactinemia (Chang, 1983; Archer, 1988).

4.1 Hyperprolactinemia and primary hypothyroidism.

Jackson (1956) was the first who described symptom triads of primary hypothyroidism, amenorrhea and galactorrhea in hyperprolactinemic patients. These were found in many cases later and further reported (Dowling *et al.*, 1961; Ross and Nusynowitz, 1968; Kinch *et al.*, 1969; Edwards *et al.*, 1971; Boroditsky and Faiman, 1972; Kleinberg *et al.*, 1977; Fish and Mariash 1988). Galactorrhea and amenorrhea usually develope in post-partum women, in those on a long-term oral contraceptive therapy and in post-thyroidectomized women (Shearman and

Turtle, 1970; Edwards *et al.*, 1971; Boroditsky and Faiman, 1972). In some cases, primary hypothyroidism without galactorrhea is revealed because galactorrhea is an uncommon accompaniment of primary hypothyroidism (Toft *et al.*, 1973; Honbo *et al.*, 1978). A positive significant correlation between TSH and PRL is reported (Onishi *et al.*, 1977; Honbo *et al.*; 1978). It is suggested that the elevated serum PRL levels of patients with primary hypothyroidism are mediated by feedback release of thyrotropin-releasing hormone (TRH) (Onishi *et al.*, 1977; Fish and Mariash, 1988). Numerous investigators have demonstrated in several species, including humans (Jacobs *et al.*, 1971; Sachson, 1972; Noel *et al.*, 1974; Kikuoka *et al.*, 1980), rats (Blake, 1974; Harris *et al.*, 1978), sheeps (Fell *et al.*, 1973) and rhesus monkeys (Pavasuthipaisit *et al.*, 1983) that TRH stimulates the secretion of TSH and PRL. Moreover, TRH is capable of enhanced PRL secretion in human pituitary cell culture (Tashjian *et al.*, 1971). Thyroid hormone replacement results in a decrease in PRL response to TRH in most patients with consequential resumption of normal menstrual cycle and cessation of milk secretion. (Boroditsky and Faiman, 1972; Snyder *et al.*, 1973; Refetoft *et al.*, 1974; Onishi *et al.*, 1977). Furthermore, elevated serum TRH levels have recently been reported in subjects with primary hypothyroidism (Montoya *et al.*, 1975). In untreated hyperthyroid patients, the response of PRL to TRH is markedly attenuated and restored to normal values

after the treatment (Yamaji, 1974). It is of interested that both the TSH and PRL response to TRH are inhibited by 3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa), a metabolite precursor of dopamine (Rapoport *et al.*, 1973; Refetoff *et al.*, 1974; Besses *et al.*, 1975; Burrow *et al.*, 1977). These authors believed that dopaminergic neurons,through the pituitary portal system, may play a role in the regulation of TSH and PRL secretion. If a common regulatory mechanism controlling TSH and PRL secretion is involved, the pituitary thyrotrophs and lactotrophs could have shown differential sensitivity to certain common stimulatory and inhibitory substances (Refetoff, 1974). Hence, the dopamine and TRH are antagonistic in their effects (Burrow *et al.*, 1977). Further findings also suggest that dopamine of hypothalamic origin serves as a physiological prolactin inhibitory factor (PIF) (Ben-Jananthan, 1980).

Scanlon *et al.*, (1977) reported a significantly larger release of TSH in hypothyroid patients than those of euthyroid patients to dopamine receptor-blocking drug, metoclopramide. Therefore, they believed that inhibition of dopaminergic system may regulate TSH release in human. In rats, two dopamine agonists (apomorphine and bromocriptine) are capable of significant suppression of TRH induced TSH secretion, and the effect is reversed by giving dopamine antagonist (pimozide) (Ranta *et al.*, 1977). Either treatments with 2L- brom-ergocryptine (CB-

154) or the stimulated dopamine receptor drugs in galactorrhea-amenorrhea syndrome in prompt resumption of normal menstruation and cessation of milk secretion (Miyai *et al.*, 1974; Boyd and Reichlin, 1975) but failed to affect the primary hypothyroidism (Christopoulos, 1986). In 1980, Feek and his coworkers reported that dopamine receptor blocker (metoclopramide) induced the rise in serum TSH and PRL concentrations and significantly greater in subclinical hypothyroid paients (normal serum T4 and raised TSH levels) compared to that in overt hypothyroid patients (low serum T4 and raised TSH). They suggested the existence of unrecognized feedback effect of thyroid hormones at the hypothalamus, resulting in increased dopaminergic inhibition of TSH release, additionally, the established negative feedfack of thyroid hormones at the level of anterior pituitary thyrotropes. Furthermore, TRH-induced release of PRL is independent of extracellular Ca^{2+} but associated with an increased release of intracellular Ca^{2+} and incorporation of ^{32}P into phosphatidyl inositides (PI) (Rillema, 1980; Gershengorn, 1985, McNeilly, 1986). This latter event induces formation of diacylglycerol and arachidonic acid following activation of the phosphatidyl inositol cycle within pituitary lactotroph. Dopamine reduces the effect of TRH on phosphate incorporation and PRL release but following the acute dopamine withdrawal enhances the sensitivity of the lactotrophs to TRH stimulation (McNeilly, 1986).

4.2 Hyperprolactinemia and amenorrhea.

Unlike normal patterns in human and non-human primates, a peak of PRL coincides with preovulatory surge of LH and occurs in laboratory rodents including rats (Neill *et al.*, 1971), mice (Kwa *et al.*, 1967) and hamsters (Bast and Greewald, 1974). Since E_2 enhances PRL secretion in the rat, it seems probably that the pro-estrus rise of PRL occurs as a result of the increase in pro-estrus E_2 secretion (Amenomori and Meites, 1970). In sheep, a rise in PRL occurs after luteal regression and coutinues until the end of the ovulatory LH surge (Cumming *et al.*, 1972). It has been suggested that the pro-estrus rise in PRL in the sheep, as in the rat, is related to the increasing levels of E_2 during the periovulatory period. But close observation of the time course of release of PRL in relation to the secretion of E_2 suggests that the increase in PRL occurs before any dramatic change in E_2 alevels (McNeilly, 1980 a) . During this initial periovulatory period, the pulse frequency of LH increases around the same time as the increase in PRL. Since dopamine appears to be inhibited to GnRH release, an increase in GnRH release may require a decrease in hypothalamic dopamine turnover (McNeilly, 1980 a ; 1986). Thus, an increase in pulse frequency of LH may reflect a decrease in hypothalamic dopaminergic activity, consequently, the change in PRL occurs and not as a cause of this decreased turnover (McNeilly, 1980 a) ; Ben-

Jonanthan, 1980). In rhesus monkeys, PRL levels during the follicular phase is significantly lower than that during the luteal phase and stage of menstruation has no effect on the nyctohemoral rhythm as judged by 24 hours profiles of serum PRL (Quadri and Spies, 1976). In women, non-systemic changes in PRL levels occur during the course of the menstrual cycle with the highest levels being either during the ovulatory period or the luteal phase (Ehara *et al.*, 1973; Franchimont *et al.*, 1976). However, a significant positive correlation between an increase in PRL levels and E_2 during pre-ovulatory period and luteal phase as compared with the follicular phase in women is shown (Franchimont *et al.*, 1976). In the periovulatory period of the menstrual cycle, infusion of dopamine into normal women causes a dramatic decrease in the levels of LH (Judd *et al.*, 1978). While basal levels of PRL were not altered significantly between early and mid cycle, the inhibitiion of PRL release by dopamine is correlated with endogenous E_2 levels. Hence, Judd and his coworkers (1978) concluded that "the selective hypersensitivity of both LH and FSH to dopamine (DA) observed on the day before the mid cycle LH peak is consistent with a reduction in GnRH neuronal inhibition by tubero-infundibular DA neurons at this time". It is clear that both in women and sheep, the released hypothalamic turnover of DA in the periovulatory period is similar. Therefore, it is probable that the consequent change in

PRL around the time of the LH surge is related not only to the increased ovarian E_2 secretion but also to a decrease in dopamine secretion (McNeilly, 1980 a .

4.2.1 Hyperprolactinemia and ovarian function

Direct evidences of an inhibitory effect of high levels of PRL are obtained from *in vitro* experiments with granulosa cell. Increasing amounts of PRL added to the culture medium of human granulosa cells inhibited progesterone secretion in a dose dependent manner (McNatty *et al.*, 1974). But PRL exerts well established stimulatory effects both *in vitro* and *in vivo* of rat follicular cells (Gibori and Richards, 1978). However, in the porcine granulosa cell, PRL action is bipotential, depending critically on the degree of granulosa cell differentiation attained *in vivo* (Veldhuis and Hammond, 1980). PRL suppresses steroidogenesis by cultured granulosa cell isolated from immature (1-2 mm) follicles but stimulates progesterone secretion by granulosa cell collected from mature (> 6 mm) follicles (Veldhuis and Hammond, 1980). It seems probable that estrogens may play an important part in regulating these divergent actions of PRL in the ovary (Veldhuis and Hammond, 1980; McNeilly, 1980(b) ; McNeilly, 1982). Since E_2 is a key factor on regulation of growth and differentiation of granulosa cell, any suppression of estrogen secretion would lead to decrease in granulosa cells population and subsequently lessened steroidogenic potential in the corpus luteum

(Richards, 1980). Moreover, McNatty (1979) has shown that elevated concentrations of PRL in plasma and human ovarian follicular fluid are associated with a reduced number of granulosa cells with an inadequate capacity to produce steroids and a marked reduction in the circulating estrogen levels. Ovulation with normal luteal function can be induced in hyperprolactinemic states by appropriate gonadotrophin treatment in sheeps (MeNeilly *et al.*, 1980a), women (Kemmann *et al.*, 1977) and rhesus monkeys (Knobil *et al.*, 1980). The follicular development is delayed in hyperprolactinemic monkeys in the face of normal levels of gonadotrophins and E_2 suggests an inhibitory action on the follicular compartment of the ovary as well (McNeilly *et al.*, 1982). In women with hyperprolactinemia, GnRH-induced release of gonadotrophins will induce normal E_2 secretion from the ovary (Caro and Woolf, 1980) and no differences in the amounts of exogenous gonadotrophins required to induce ovulation (Fraser *et al.*, 1978; McGarrigle *et al.*, 1978).

It is widely known that estrogens induce an increase in PRL levels in blood which usually reported in the women continually taking contraceptive pill (Edwards *et al.*, 1971; Boroditsky and Faiman, 1972; Honbo *et al.*, 1978). Estrogens appears to decrease hypothalamic dopamine release by inhibiting the activity of tyrosine hydroxylase leading to a decrease in conversion of tyrosine to L-dopa, the immediate precursor of dopamine,

with the resultant of increase in PRL secretion (McNeilly, 1986). It is observed in rats that the corpus lutea of pregnancy and pseudopregnancy are critically dependent on PRL until D8 and will not function unless PRL levels remain elevated during this time (Garris and Rothchild, 1980). Normal luteal function occurs after ovulation in spite of elevated levels of PRL in daily cows (Peters *et al.*, 1979; Carruthers and Hafs, 1980), while in rhesus monkeys with lesions of the arcuate nucleus and associated hyperprolactinemia, corpus luteum function is normal after ovulation induced with pulsed injection of LH-RH (Knobil *et al.*, 1980). Recently, Richardson and his colleague (1985) reported that markedly elevated PRL levels which induced by hypothalamic lesions did not influence the time courses of postovulatory plasma progesterone concentration compared to those in monkeys with normal PRL concentrations. Nonetheless, the plasma progesterone remained the elevated levels at the end of luteal phase therefore, PRL in high concentrations could partially maintain established corpus luteum (Richardson *et al.*, 1985). In women, hyperprolactinemia usually associates with short or deficient luteal phases (Seppala *et al.*, 1976; Muhlenstedt *et al.*, 1977). Indeed, when hyperprolactinemia is induced by pharmacological agents in the luteal phase of the menstrual cycle (TRH by Jewelewicz *et al.*, 1974; sulpiride by Robyn *et al.*, 1976) or when bromocriptine therapy in hyperprolactinemic patients is

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hyperprolactinemia syndrome.

4.3 Hyperprolactinemia and galactorrhea

Galactorrhea is an uncommon accompaniment of primary hypothyroidism, when present, is not necessarily associated with elevated serum PRL levels (Toft *et al.*, 1973, Onishi *et al.*, 1977; Honbo *et al.*, 1978). Factors that facilitate the development of galactorrhea are the post-partum state, oral contraceptive administration and estrogen-progesterone priming of the breast (Edwards *et al.*, 1971; Besser and Edwards, 1972; Boroditsky and Faiman, 1972; Onishi *et al.*, 1977; Honbo *et al.*, 1978). Most patients suffered from hyperprolactinemia with galactorrhea always accompany with amenorrhea and infertility (Boroditsky and Faiman, 1972; Kleinberg *et al.*, 1977; Frantz, 1978; Archer, 1980). In these patients, increased serum PRL levels undoubtedly promote the continuation of galactorrhea. However, it is unadequate by itself to initiate the lobular-alveolar differentiation and development (Honbo *et al.*, 1978; Archer, 1980). It is widely known that during pregnancy in most higher primates including man, plasma PRL levels increase steadily and reach a peak in late pregnancy near the time of delivery (Shiu and Friesen, 1980, Schallenberger *et al.*, 1981; McNeilly, 1986). During pregnancy the milk secretion, lobulo-alveolar structure of the mammary gland develops under the sequential stimulation of estrogen, progesterone and placental

lactogen from the ovary and the placenta, pituitary growth hormone, PRL and adrenocortisol steroids (Archer, 1980; McNeilly, 1986). PRL is very important hormone for stimulation of milk secretion and protein synthesis in most mammals (McNeilly, 1986). TRH induced hyperprolactinemia in women results in marked breast engorgement and milk let-down (Tyson *et al.*, 1972). In men whose breasts have not been subjected to expose with estrogenic and progestational priming, hyperprolactinemia rarely leads to galactorrhea (Frantz, 1978). After treatment with bromocriptine, the levels of PRL are immediately decreased and accompanied with reduction of galactorrhea (Edwards *et al.*, 1971; Besser and Edwards, 1972; Noel *et al.*, 1974; Boyd and Reichlin, 1975; Kleinberg *et al.*, 1977; Onishi *et al.*, 1977; Honbo *et al.*, 1978; Duchen and McNeilly, 1980).

5. A primate model of study the influences of maternal hypothyroidism upon the fetus

The study of the effects of iodine deficiency in human are limited by ethical consideration. These apply both to the setting up of controlled or observed trials including availability of post mortem specimens. In these circumstances, experimental animal models have been developed for investigation of the observed mechanisms.

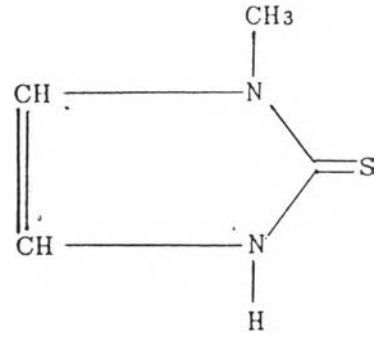
Studies of thyroid hormones function in monkeys are relatively rare. Thyroid gland of the fetal rhesus

monkey is actively trapping and incorporating iodide and synthesizing T4 and its precursors, monoiodothyronine (MIT) and di-iodothyronine (DIT) by day 75 of gestation while follicle formation and colloid synthesis are apparent in the gland by day 50 of gestation (Pickering, 1961a; 1961b; 1968). Pronounced increase in MIT, DIT and T4 with advancing conception age is noted (Pickering, 1961a). After birth, thyroid gland contents of these components do not show a considerable increase with advancing age. Approximate 15-20 ug L-thyroxine/Kg/day is needed for normal metabolism, growth and development during neonatal life (Pickering, 1961b). Tyson and his coworkers (1972) reported the fetal abnormality, ie. skeletal growth retardation, occurred when injected intravenously with ^{131} 2 m Ci/Kg BW. to the mother on day 71-88 of gestation. After introducing radioimmunoassay method for determination of thyroid hormones in blood relative to the human standard and antiserum, the investigations of thyroid hormones functions are widely reported in the monkeys. Administration of thiouracil to rhesus monkeys (0.1 - 0.4 gm/day) cause hyperplasia and colloid depletion as it does in other animals and in man (Engle and Aranow, 1946). The interactions of thyroid-pituitary-hypothalamic axis in the rhesus monkey is very similar to those in human (Belchetz *et al.*, 1978 b). T3 is able to inhibit rapidly TSH secretion by a direct action upon the hypothalamus (dorsomedial nucleus and

lateral hypothalamic area extending into the preoptic region) (Belchetz *et al.*, 1978b). Basal values of serum T3, T4 and TSH are slightly different from other scattered reports (Azukizawa *et al.*, 1976; Belchetz *et al.*, 1978 a, Kaack *et al.*, 1979; Melmed *et al.*, 1979; Smallridge, 1981; Kamis, 1982; Ren *et al.*; 1988, Suwanprasert *et al.*, 1988; 1989; 1990; Varavudhi *et al.*, 1988; 1989). It is of interested that the basal values of thyroid hormones in new world monkeys (Squirrel monkey) are less than those in the old world monkeys (rhesus monkey, african green monkey, talapoin monkey and chimpanzee) while T3:T4 ratio in female is higher than those in male in all of these animals (Kaack *et al.*, 1979). In addition, TRH is able to stimulate PRL secretion in monkeys and is similar to man (Jacobs *et al.*, 1971; Azukizawa *et al.*, 1976). Moreover, fetal thyroid of the rhesus mokey during the latter period of gestation can release both T3 and T4 in response to TSH (Azukizawa *et al.*, 1976). Similar patterns of circadian variations in serum thyroid hormones in man (Refettof *et al.*, 1970), cynomolgus monkeys (Kamis, 1982) and rats (Jordan *et al.*, 1980) are also demonstrated. However, little is known about the effect of maternal iodine deficiency on the fetus in primates. Successful induction of iodine deficiency in marmoset is only the first case report by feeding with low iodine diet (14.9 $\pm$ 0.8 ng iodine/gm moist diet) for over 6 months (Mano *et al.*, 1985; 1987). The low iodine status has been confirmed by low plasma T4 and high TSH levels. The mechanism of

iodine deficiency in marmoset is mediated through hypothyroidism which similar to those in human (Pharoah *et al.*, 1971; 1983; Mano *et al.*, 1985; 1987; Hetzel and Mano, 1989). The kinetic study of thyroid hormone in male cynomolgus monkey as following : metabolic clearance rate (MCR), production rate (PR) and the half-life of elimination (t1/2 B) are 21.5 ± 0.6 ml/kg/day, 1.34 ± 0.23 ug/kg/day, 29.6 ± 2.0 hr for T4 and 156.6 ± 12.0 ml/kg/day, 0.33 ± 0.04 ug/kg/day and 13.3 ± 1.3 hr for T3, respectively (Smallridge *et al.*, 1981).

There are four categories which can inhibit the thyroid hormone functions by using antithyroid drugs : ionic inhibitions, excess iodide and radioactive iodine. 1, methyl-2-mercaptoimidazole or methimazole (MMI) is one of the thioureylenes compound, MW 480, having the thiocarbamide group (S=C-N) being essential for antithyroid activity (Kampmann *et al.*, 1981; Hallengren *et al.*, 1982; Jansson *et al.*, 1983; Cooper, 1984; Heynes and Murad, 1985).



MMI effectively inhibits iodination of tyrosyl

residues in thyroglobulin and interferes with iodination by acting as substrates for the postulated peroxidase-iodinium complex, thus competitively inhibiting the interaction with tyrosine (Taurog, 1976; Kampmann *et al.*, 1981). Daily dose of MMI requires for initiation and maintenance of thyrotoxicosis are 30-60 mg and 5-20 mg/day, respectively (Solomon, 1978). The minimum effective dose of 15 mg/day given as the single dose is further reported (Shiroozu *et al.*, 1986). The clinical effects of antithyroid drugs are related to their concentration in the thyroid gland rather than in the plasma (Lazarus *et al.*, 1975), thus, intrathyroidal concentration of MMI after carbimazole ingestion (prodrug of MMI) is maintained high at least for 20 hours in adult man (Jansson *et al.*, 1983 a) and in children for 16-24 hours (Okuno *et al.*, 1987). Moreover, the metabolic turnover in the thyroid is slower than in plasma (Jansson *et al.*, 1983 b). Several pharmacokinetics studies on MMI have already presented, but the results are not always consistent due to the different methodological approaches. Pittman *et al.* (1971) reported in adult human, the peak plasma levels of 8.1 umol/l was obtained 1 hour after ingestion of 60 mg MMI with a half-life of 6.4 hours by calorimetric measurement. With high performance liquid chromatography, Skellern *et al.* (1980) reported peak values of 17.5 to 26.3 umol/l at 0.5-1 hour, half-life of 2.96 hours after 60 mg of MMI ingestion. But Melander and

his coworkers (1980) used this method and reported the peak value of 4.64-21.55 umol/l at 1-3 hour and the half-life was 2-7 hours. By using the RIA method, Cooper *et al* (1984) reported peak concentrations of MMI ingestion were 6.8 and 11.8 umol/l after 30 and 60 mg/day in hyperthyroidism and the half-life was 6.8 hours, respectively. Recently, Jansson *et al.*, (1985) reported ingestion of 10 mg MMI had an initial distribution half-life of 0.10-0.23 hours and elimination half-life of 4.9 to 5.7 hours by using gas chromatographic mass spectrometric assay. No difference in elimination half-life is seen among euthyroid, hypo and hyperthyroid patients (Janssan *et al.*, 1985). The bioavailability (F) of MMI is reported as either 93% (Jansson *et al.*, 1983; Jansson *et al.*, 1985) or 81-99% (Kampmann *et al.*, 1981). Only one case report using MMI induced-hypothyroidism in cynomolgus monkey was the study of Ren and his colleagues (1988). They fed the animal with 0.0125% MMI in drinking water for a minimum of 12 weeks in order to establish the hypothyroid state.