

THE EFFECTS OF AMINO ACIDS ON FOLLICULAR AND TESTICULAR FUNCTION

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ABSTRACT OF THESIS

The aim of this work was to measure the effect of Serine/Threonine on the follicle (in terms of growth, differentiation, atresia and the expression of genes for TGF β 's and steroidogenic enzymes) and on the testis (in terms of seminiferous tubular diameter, Leydig cell diameter, number of spermatids and expression of genes effecting hormonal balance within the testis). An additional aim was to measure the effect of Serine/Threonine on gonadotrophin secretion and gonadotrophin response to GnRH or a GnRH analogue.

Six experiments were conducted. In the first experiment, preantral follicles, from 21 day old female mice were dissected mechanically and cultured individually for seven days with or without Pregnant Mare Serum Gonadotrophin (PMSG) to examine the effect of the amino acids on follicle growth. The addition of a mixture of Serine/Threonine (with PMSG or without PMSG) increased follicle growth, but not significantly.

In the second, 19-21 day old female were injected with saline (Control) or Serine/Threonine (Test), for 5 days to test the effect of these amino acids on follicle growth and atresia. Amino acid injection significantly decreased the number of primordial follicles entering the primary stage, but after the primary stage, growth rate was greater in amino acid injected animal. The number of pre-antral, early and late atretic follicles was greater in the amino acid group. Amino acids significantly increased the number of atretic preantral follicles. More follicles, in test group, went on to the antral stage but in the antral stage they became atretic.

In the third experiment, 19-21 day old male mice were injected with amino acids (using the protocol as in experiment 2). The injections decreased the number of spermatids (2- 3 stage), but the differences were not significant. In amino acid injected animals, tubule diameter and the diameter of Leydig cells was greater (not significant) than that of controls.

In the fourth experiment, Sheep were injected intravenously with a mixture of Serine/Threonine (AA) and /or saline (S) with or without injection of GnRH to test the effect of these amino acids on pituitary response to GnRH. In the fifth experiment,

sheep were injected with different doses of GnRH or a GnRH analogue (azagly-nafarelin) to confirm if azagly nafareline was a potent analogue of GnRH.

The injection of amino acids before or together with GnRH caused a significant increase in LH secretion whereas the secretion of FSH was suppressed. Injection of GnRH or GnRH with amino acids caused a significant increase in LH secretion, whereas the secretion of FSH did not change. Injection of sheep with different doses of GnRH or a GnRH analogue showed that small doses of GnRH or GnRH analogue were not effective in causing LH or FSH secretion whereas higher doses led to pulses of LH and FSH. The data also show that the analogue is approximately 38 times more potent than GnRH.

In the sixth experiment, male and female mice were injected with Serine/Threonine for 5 days to measure the expression of mRNA for TGF- $\beta_{1,2,3}$, LHR, FSHR, P450_{scc} and P450_{arom}. The amino acid injection increased the expression of genes for TGF- β 's within the ovary and testis. Expression of genes for LH and FSH receptors and steroidogenic enzymes were decreased in the female while they were increased in the male.

The results shows that; Injections of Serine and Threonine amino acids increased follicle growth and atresia while the number of spermatids was decreased in testis. The result shows that injecting a mixture of Serine and Threonine is capable of altering the gonadotrophin secretion and the expression of TGF β like proteins within testis and ovary.

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CHAPTER I; INTRODUCTION

The aim of animal production systems is to convert plant products into animal products that can be used by man (Greenhalgh, 1992). World population is increasing very rapidly. Between 1950 and 1988, the human population of the world doubled from about 2.5 billion to more than 5 billion. The increase in population is greater in developing countries. By the year 2000, the proportion of the world population living in the developing world will have increased from 73% to 78% (Bunting, 1992).

The main concern is that the growth in world population will eventually overrun the capacity of the farming environments of the planet to produce sufficient food. This concern has led humans to seek solutions by developing new agricultural techniques through science, to increase food production per head in both developing and developed countries. Hopefully, this worldwide process of agricultural improvement, based on research will result in more productive and profitable methods of crop and livestock production (Lister, 1992).

Advanced techniques in animal reproduction are needed to preserve and increase the population of genetically superior animals required to improve the quality and quantity of food of animal origin. Examples are the development of superovulation techniques, recovery of primordial and preantral follicles for *in vitro* production of oocytes and *in vitro* fertilisation (Van den Hurk *et al.*, 1996).

To understand the basic factors affecting follicle growth, oestrus, ovulation, fertilisation and embryo survival scientists have used *in vivo* and *in vitro* methods. Hormones and drugs have been used to control reproduction, examples are the synchronisation of oestrus, especially in sheep, by progesterone releasing devices (CIDR) and the prostaglandin analogue, cloprostenol and the use of Gonadotrophin Releasing Hormone (GnRH) analogues and gonadotrophins (LH, FSH and PMSG) to stimulate ovarian function.

The development of mouse follicle culture was evidence that follicle growth was possible *in vitro* (Ryle 1971a; Ryle 1971b). Experiments on freshly dissected large follicles from various species showed that oocyte maturation and ovulation could be induced *in vitro* (Hillensjo, 1976). Many studies on isolated and purified cell types

have identified factors that might influence follicle development. More recently, various methods of growing small intact follicles to maturity *in vitro* have been developed (Nayudu *et al.*, 1990; Boland *et al.*, 1991; Nayudu and Osborn, 1992; Hardstone and Clark, 1992, Boland *et al.*, 1993).

The reproductive performance of an animal is influenced by its genotype and its environment and nutrition is a major environmental factor effecting reproduction. The nutritional mechanisms affecting ovulation rate are not completely understood. It was postulated that the effect of nutrition on ovulation rate was indirect, acting through the hypothalamus-pituitary axis to alter secretion of gonadotrophins, an alternative hypothesis is that the effect of nutrition can be direct, acting on the follicle to modulate the action of gonadotrophins (Scaramuzzi, 1994). Many experiments have attempted to determine the nutritional mechanisms effecting ovarian function. It was suggested by Nottle *et al.*, (1988), that increased ovulation rate in sheep was the result of protein digested post-ruinally, but it has not been determined whether responses, in ovulation, to protein *per se* or the result of increased uptake of one or more amino acids, while Teleni *et al.*, (1989), suggested that nutritional increases in ovulation rate, were the result of increases in the supply of energy yielding nutrients (eg. Glucose and acetate).

Attempts to investigate effect of energy and protein on ovulation rate include the feeding lupin grain, the injection of energy yielding substrates (e.g. glucose and acetate) and the modification of rumen fermentation to increase propionate production using the ionophore, lasalocid or by feeding ruminally undegradable protein (e.g. Formaldehyde treated casein). High protein diets have been shown to affect gonadotrophin and steroid secretion as well as hepatic function. Smith, (1988), reported that plasma concentrations of FSH and androstenedione of sheep were increased while the concentrations of LH were not by a high protein diet fed around the time of luteolysis. High protein diets increased hepatic oxidative enzyme activity in the liver and this was proposed as a mechanism by which nutrient intake may influence ovulation. Phenobarbital is known to increase cytochrome P-450 enzyme activity in the liver. Administration of phenobarbital to sheep, in the pre-mating period significantly increased hepatic enzyme concentration when the sheep were on low protein diet (Smith *et al.*, 1990) and also increased ovulation rate.

Scaramuzzi, (1994), suggested that since metabolic hormones such as insulin, glucagon, IGFs, growth hormone, prolactin, thyroxin and some gut hormones are all responsive to changes in nutrient supply any one, alone or in combination with others could modulate nutritional effects on ovulation rate by acting directly on the ovary to influence follicle development and/or follicle selection. Specific metabolites or metabolic hormones reflecting the nutritional status and the availability of oxidizable metabolic fuels (glucose, amino acids and non-esterified fatty acids) may influence activity of neurones required for GnRH secretion (Schillo, 1992). A diet known to increase gonadotrophin secretion also caused an increased concentration of metabolic fuels and metabolic hormones (Miller, 1998). Blache *et al.*, (1996) have suggested that increased ovulation rate, because of diet, is not entirely a result of the protein and energy content of diet, and that specific components of protein and energy are also important. Downing *et al.*, (1995); suggested that dietary amino acids which are precursor for neuro transmitters may be important in the mechanisms of nutritional effects on ovulation rate. Therefore the effects of the amino acids (e.g. L-Arginine, the branched chain amino acids and the excitatory amino acids) on gonadotrophin secretion have been extensively investigated (Downing *et al.*, 1995a; Recabarren *et al.*, 1996; Honaramooz *et al.*, 1998).

The effects of amino acids on ovarian or testicular function are not entirely the result of their being precursors for the neurotransmitter synthesis effecting the secretion of gonadotrophins, but they may also be the result of expression of new proteins within the testis and ovary regulating follicular growth, differentiation, spermatogenic function and hormone secretion due to the phosphorylation of Serine and Threonine in proteins taking the role in gene transcription and cell signalling. Serine and Threonine amino acids are constituted by these proteins and phosphorylated by protein kinases. This phosphorylation is important for the production of new proteins (eg TGF- β) and hormone secretion effecting gonadal function.

Some of the intra-cellular proteins that cause gene expression and hormone secretion are phosphorylated on Serine and Threonine residues, examples are the cAMP-responsive element binding proteins (CREB) and the cAMP-responsive enhancer element modulator (CREM) (Potchinsky *et al.*, 1997). Steroidogenesis Inducing Protein (SIP), isolated from human follicular fluid stimulates growth and

steroid production of Leydig cell from immature rats (Khan *et al*, 1993). It has a mechanism of action similar to that of the majority of growth factors and increases phosphorylation of Serine and Threonine within the intracellular proteins. All of the induced-proteins isolated, from SIP-stimulated-leydig cells are phosphorylated on Serine and Threonine residues (Khan *et al*, 1993).

The phosphorylation of Serine and Threonine may alter the expression of genes for the TGF- β like proteins in the ovary and testis thus effecting gonadotrophin response, hormone production, follicle growth, differentiation and atresia. Therefore the objective of this thesis was to determine effect of Serine and Threonine administration on follicular growth, spermatogenic function, gonadotrophin secretion and gene expression for TGF- β , the gonadotrophin receptors and some steroidogenic enzymes.

CHAPTER II; LITERATURE REVIEW

2.1. FOLLICULOGENESIS IN SHEEP

Folliculogenesis is the process of follicular growth and development (Figure 2.1.1. and Figure 2. 1. 2.). This process begins with the formation of primordial follicles (Sidis *et al.*, 1998). Primordial follicles are formed before birth and are essentially quiescent, they contain an oocyte surrounded by pre-granulosa cells contained within a basement membrane (Scaramuzzi *et al.*, 1993). Early follicular development is characterised by increases in the size of oocytes and the number of layers of granulosa cells surrounding the oocyte (Moor and Walters, 1979; Findlay, 1993).

The ovary of one ewe lamb contains about $230,000 \pm 120,000$ primordial follicles and only 250-1500 of them have start to grow (Mariana *et al.*, 1991).

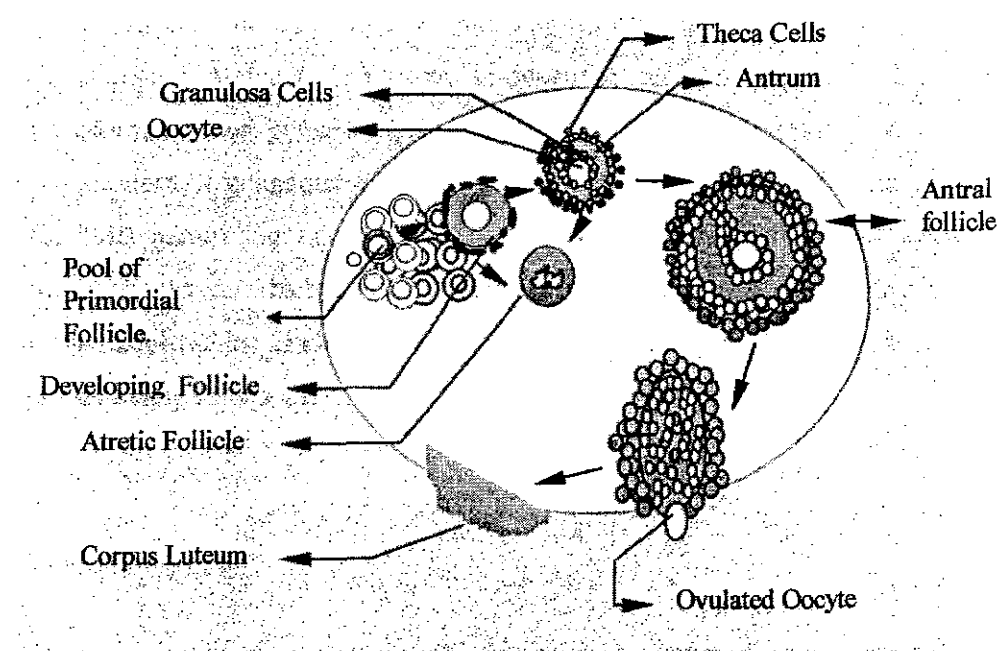


Figure 2. 1. 1. ; The ovary of an adult ewe contains a definitive population of primordial follicles and smaller numbers of follicles at various stages of growth and development. After release of an oocyte from a follicle, the basement membrane between theca interna and membrane granulosa begins to break down and blood vessel invade the antral space causing extensive vascular development and the formation of the corpus luteum (Modified from Scaramuzzi *et al.*, 1993 and Hutchinson, 1993).

From the start of development of primordial follicles to ovulation takes an estimated 180 days, in sheep (McNeilly *et al.*, 1991). Only a limited number of follicles can leave the primordial pool at the same time to enter the growth phase. During the oestrus cycle, 3-4 follicles/day enter the preantral growth phase. Once a follicle is recruited from the pool of primordial follicle, RNA synthesis within the oocyte, mitotic activity and cuboidal development of pre-granulosa cells leads to the formation of egg membrane (zona pellucida). Follicles at this stage are named as committed follicles. A committed follicle develops receptors for both FSH and LH on granulosa cells, and it becomes responsive to LH and FSH and can synthesize cAMP and steroids. Follicles in this stage are named as gonadotrophin-responsive follicles. The FSH requirement of gonadotrophin-responsive follicles increases but FSH levels are reduced. There is an increased rate of atresia. The induction of LH receptors on granulosa cells of these follicles and increased aromatase activity at this stage of development produces gonadotrophin-dependent follicles (Figure 2.1.2.). During the transformation of gonadotrophin-dependent follicles to ovulatory stage, the FSH requirement of gonadotrophin-dependent follicles is reduced but, it is still important for final maturation of granulosa cells. The number of granulosa cells with LH receptors increases to maximum levels and the follicles become extremely responsive to gonadotrophins leading to increased secretion of oestradiol and inhibin which consequently results with oestrus and ovulation.

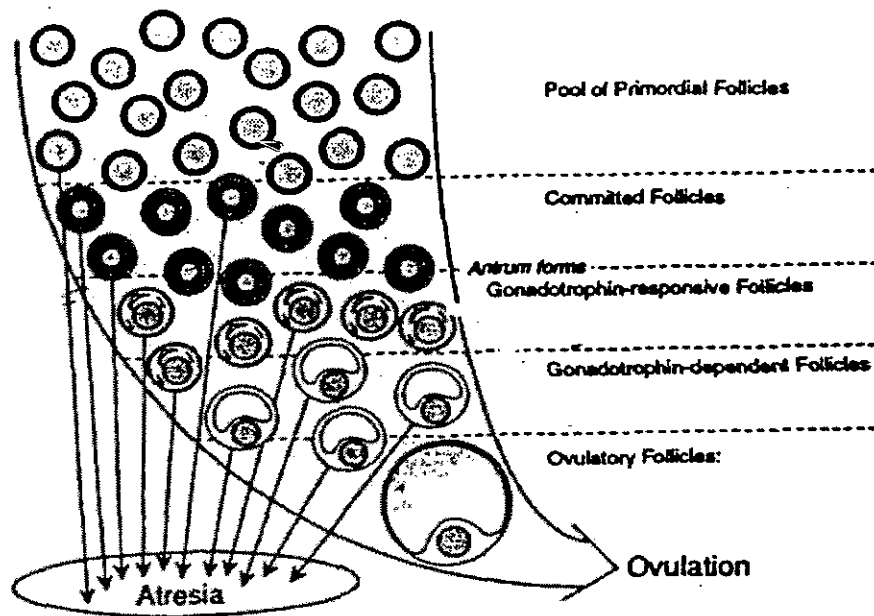


Figure 2. 1. 2. ; Primordial follicles are formed at around day 100 of fetal life (McNeilly *et al.*, 1991). Follicles leave the primordial stage of development in an ordered sequence. The first stages of follicular growth progress more slowly than the final stage of follicular development (Fortune, 1994). Once a follicle is recruited from the pool of primordial follicles, development continues until either atresia or ovulation occurs. There is little atresia in primordial follicles; it is also rare in follicles smaller than 0.5-1mm (Modified from Scaramuzzi *et al* 1993).

The mitotic index of cells within a follicle varies depending on its stage of development (Baird and McNeilly, 1981). It takes 34-43 days for a follicle to grow from the end of the pre-antral stage to the ovulatory stage. During the first 25-34 days of this time, follicle diameter reaches 0.5 mm, in a further 5 days follicle diameter reaches 2.2 mm and in another 4 days follicle diameter reaches 4-5 mm (Cahill, 1984; Downing and Scaramuzzi, 1991). Therefore, the initial stages of follicular growth proceed much more slowly than the final stages (Fortune, 1994; Fehrenbach *et al.*, 1998).

It is still not known what mechanism causes primordial follicles to enter the growth phase and where the initial stimulus comes from. It was suggested that the entrance of primordial follicles into the growth phase was gonadotrophin dependent.

This suggestion was not confirmed by experiments in hypophysectomised sheep, because hypophysectomy did not inhibit primordial follicles from entering the growth phase. It just caused a significant decrease in the numbers of non-atretic pre-antral follicles after 70 days (Al-Gubory *et al.*, 1987). Prolactin has been shown to also have a role in the initiation of follicular growth and prolactin receptors have been observed in the oocyte of primordial and small follicles (Dunaif *et al.*, 1982). Treatment of sheep with bromocryptine, to reduce prolactin levels also reduced the number of growing follicles (Cahill, 1984). Thus gonadotrophins may not be essential agents in initiating follicular growth, but a basal level of FSH and LH is required for normal development (McNeilly and Fraser, 1987). In sheep, growth and development of ovarian follicles beyond 2mm is dependent on gonadotrophin support. Deprivation of gonadotrophins by hypophysectomy or hypothalamic-pituitary stalk disconnection leads to arrested growth, in follicles smaller than 2mm in diameter, and atresia, in follicles bigger than 2mm (Eckery *et al.*, 1997).

2. 1. 1. CONTROL AND REGULATION OF FOLLICULAR DEVELOPMENT AND DIFFERENTIATION.

Follicular growth and differentiation are controlled by gonadotrophins, growth factors and ovarian steroids. Follicle Stimulating Hormone and LH are glycoproteins synthesised in the same cells of the anterior pituitary gland in response to GnRH which is secreted from the median eminence of hypothalamus (Fig 2. 1. 1. 2.). The amount of gonadotrophin secreted is a function of the amount of GnRH reaching the pituitary gland and the responsiveness of anterior pituitary gland to GnRH. Steroids hormones influence the secretion of gonadotrophins by changing the frequency of release of GnRH from the hypothalamus and by altering the responsiveness of the anterior pituitary gland to GnRH (Baird and McNeilly, 1981; Kumar *et al.*, 1997). Increased sensitivity of anterior pituitary gland to GnRH alone is not capable of producing the high levels of LH seen during the preovulatory surge. Thus, an additional factor is required. This additional factor is a rise in GnRH secretion or an alteration in its pattern of secretion (Hammer, 1985; Morrell, *et al.*, 1985; Lehman *et al.*, 1986; Clarke, 1987; Picton *et al.*, 1990). Ovarian steroids (Oestrogen and progesterone) have positive or negative feedback effect on GnRH secretion depending on stages of oestrus cycle. The mode of the steroid feedback effects on GnRH

secretion is differentially regulated in the early follicular phase, the late follicular phase and in the luteal phase of the oestrus cycle of sheep (Clarke, 1987).

Both FSH and LH increase the activity of steroidogenic enzymes in granulosa and theca cells. Binding to receptors activates the adenylate cyclase-cAMP response systems that leads to activation of protein kinases and consequently phosphorylation of various proteins leading to production of steroids (Fig 2. 1. 1. 1.).

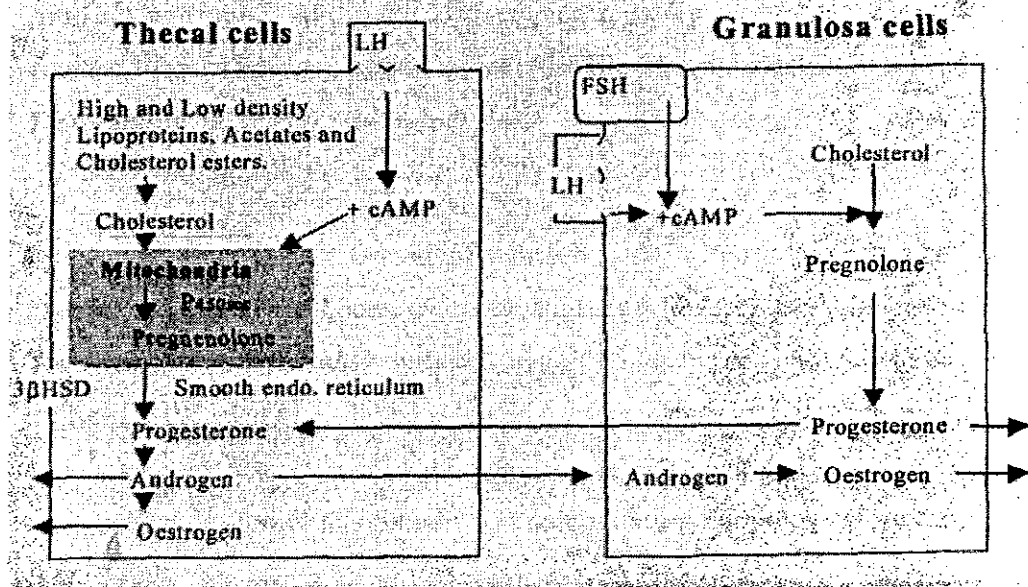


Figure 2. 1. 1. 1.; Theca cells always express receptors for LH whereas granulosa cells from small follicles only express receptors for FSH. Granulosa cells from medium and large follicles express receptors for both FSH and LH. FSH and LH increase endocytosis of lipoproteins by increasing their receptor number on follicular cells. Internalised lipoproteins are metabolised to cholesterol. The importance of high and low density lipoproteins, in steroid biosynthesis, depends on the species. In human, pig and bovine low density lipoproteins are much more important whereas in mouse high density lipoproteins contribute to a higher secretion of steroids. LH and FSH increase cAMP formation which increases mobilisation of cholesterol from lipoproteins and conversion of cholesterol into pregnenolone through activation of mitochondrial side-chain cleavage enzyme systems and the conversion of pregnolone to progesterone through activation of the 3-Beta Hydroxysteroid

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Dehydrogenase enzyme system (3β HSD) in smooth endoplasmic reticulum. Gonadotrophin stimulation also alter cellular ultrastructure. Treatment of culture of human luteal cell with LH increases number of smooth endoplasmic reticulum and the secretion of progesterone (Schmidt *et al.*, 1986). In porcine and in rat, LH and FSH stimulation of luteinizing granulosa cell resulted with the expansion of rough and smooth endoplasmic reticulum with an increased secretion of progesterone (Amsterdam *et al.*, 1988). Granulosa cells do not produce androgen. Androgen is produced only by theca cells. Granulosa cells convert thecal androgen to oestradiol (Modified from Armstrong *et al.*, 1981 and Niswender *et al.*, 1994).

Adenylate cyclase consist of two sub units, a regulatory sub unit (G or N protein) and a catalytic subunit which metabolises ATP to cyclic AMP (Ireland, 1987). The adenylate cyclase-cAMP system is important for the secretion of steroids and the mRNA for the adenylate cyclase activating polypeptide is expressed in granulosa cells of preovulatory follicles under the influence of FSH and LH (Gras *et al.*, 1996). Increased expression of this protein also reduces atresia in preovulatory follicles (Lee *et al.*, 1999).

Development of gonadal response to LH and FSH depends on the expression of LH and FSH receptors. Prenatal follicle growth is independent of FSH (O'Saughnessy *et al.*, 1997). A group of growing preantral follicles becomes responsive and dependent upon gonadotrophins especially FSH. The sensitivities of these follicles to gonadotrophins are probably determined by the number of receptors for LH, FSH and possibly prolactin. The development of these receptors depends on the stages of differentiation of the follicle. Granulosa cells of small follicles have FSH receptor but do not have LH receptors until an antrum forms (Armstrong *et al.*, 1981; Ireland 1987). The development of receptors for gonadotrophins is also influenced by the local concentration of steroid and peptide hormones within the follicles (Baird and McNeilly, 1981).

The steroidogenic potential of a follicle is effected by the presence of receptors for gonadotrophins. Small follicles produce greater amounts of cAMP in response to FSH but not LH. This is because only FSH receptors are present in these follicles. As a

consequence, there is little oestradiol secretion by these follicles. Medium sized follicles produce more oestradiol than small follicles because granulosa cells of these follicles have receptors for both FSH and LH (Armstrong *et al.*, 1981). Induction of LH receptors on granulosa cell occur when the follicle diameter reach to 1mm in sheep and 300-400µm in mice and rat (Peng *et al.*, 1991).

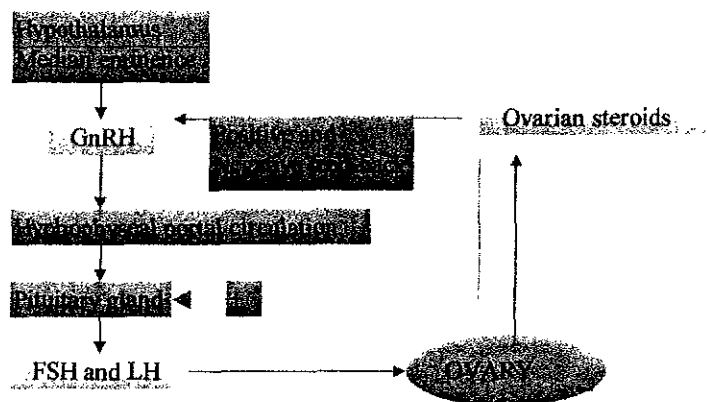


Figure 2. 1. 1. 2.; Gonadotrophin-releasing hormone (GnRH) from hypothalamus controls the secretion of LH and FSH from the anterior pituitary gland. Through this gland, the ovaries are controlled. Ovarian steroids have negative and positive feedback effect on the hypothalamus and anterior pituitary gland to modulate the secretion of FSH and LH.

2. 1. 1.1. PITUITARY GONADOTROPHIN SECRETION AND FOLLICULAR GROWTH, DEVELOPMENT AND DIFFERENTIATION.

The differences in the secretion of pituitary gonadotrophins, during the luteal phase and follicular phase of the oestrus cycle control the differentiation of preovulatory follicles (Xu *et al.*, 1989). Differentiation of preovulatory follicles is a four step process namely recruitment, selection, dominance and atresia coincident with transient increases and decreases in FSH. Recruitment establishes a group of follicles capable of ovulating. Selection occurs among these follicles but only one or two follicles are selected to complete maturation and ovulate, while the remaining undergo atresia. The selective rise in LH levels that occurs during the luteal-follicular transition is a potent stimulus for recruitment, and several early antral follicles begin to enlarge in this phase of the cycle (Marco Filicori, 1999).

Selection is a hypothetical physiological process whereby excess follicles are reduced to the ovulatory quota and dominance is a process that enables the selected follicle to suppress further growth of other follicles, escape atresia and continue to grow until ovulation or eventual atresia. Selection is accompanied by a progressive increase in the ability of theca cell to produce androgen and granulosa cells to aromatize androgen to oestradiol. A small rise in plasma LH during the follicular phase seems necessary for this change (Fortune, 1994). Dominance occurs in the follicular phase when serum concentrations of LH increase and FSH decreases (Sunderland *et al.*, 1994). LH is secreted in a pattern of pulses throughout the oestrus cycle (Martin 1984), whereas FSH secretion is continuous, but during the luteal phase FSH is secreted in a low pulsatile manner in some individual ewes (Campbell *et al.*, 1991; Findlay 1994). The FSH concentration in blood is lowest, 24-36 h after the beginning of the follicular phase and remains at this level until the preovulatory surge of LH and FSH (Baird, 1983; Scaramuzzi *et al.*, 1993). The pattern of secretion of gonadotrophins during the oestrus cycle are shown in figure 2. 1. 1. 1. 1.

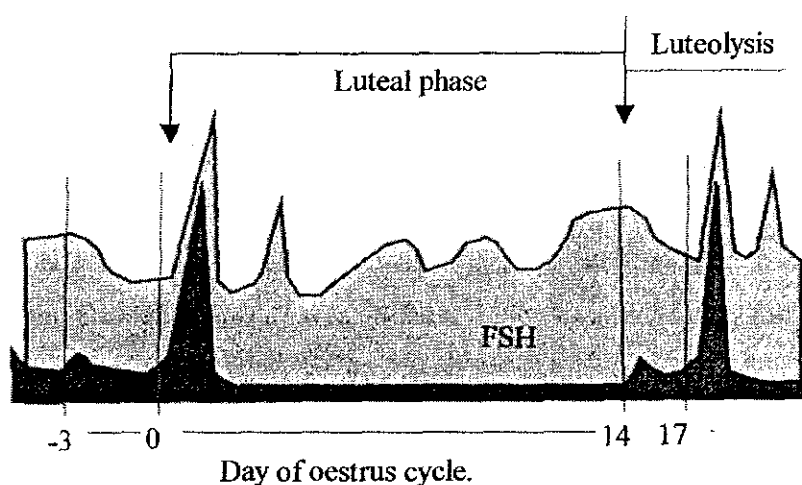


Figure 2. 1. 1. 1. 1; In adult sheep, secretion of FSH is sufficient for the development of follicles beyond the antral stage (3-5mm) at all times. The secretion of LH during the luteal phase is too infrequent and its concentration remains low during the most of oestrus cycle. In luteal phase, FSH secretion is also low, but not pulsatile. During luteolysis, the further development of follicles depends on stimulation by adequate amount of LH. As luteolysis starts, LH pulses

(Continued over page)

increases (One pulse per hour) and FSH secretion begins to decrease a few hours after luteolysis begins due to the increased secretion of oestradiol (Baird and McNeilly, 1981). Low levels of oestradiol have negative effect on FSH secretion, whereas high levels are stimulatory. Once oestradiol secretion achieves a certain level a preovulatory surge of LH and FSH occur . The preovulatory LH surge initially stimulates and then totally inhibits further secretion of oestradiol from the ovulatory follicle which causes a second small peak of FSH at about time of ovulation (Adopted from Scaramuzzi *et al.*, 1993).

The secretion of FSH and LH influence the final stages of follicular growth and development. Suppression of pituitary gonadotrophins by immunoneutralisation (McNeilly *et al.*, 1986) or by chronic treatment with GnRH agonists decrease pre-ovulatory follicle growth beyond 2.5 mm (Picton *et al.*, 1990; McNeilly, 1991c). After the suppression of gonadotrophins, infusion of FSH with basal, non-pulsatile LH, induces follicular development (Picton *et al.*, 1990) but the infusion of LH alone in a pulsatile manner, after chronic treatment with GnRH agonist, buserelin, did not appear to induce preovulatory follicle growth (Picton and McNeilly, 1991). Therefore, FSH is the principal gonadotrophin controlling the number of preovulatory follicle that grow, develop and differentiate by increasing the activity of aromatase enzyme of granulosa cells and thereby controlling the number of large antral follicle that to ovulate (McNeilly, 1991; Wang and Greenwald, 1993).

LH pulses during follicle selection directly affect the action of FSH on the follicle and play an important role in the selection of an ovulatory follicle by actively inducing atresia (Picton *et al.*, 1990; Picton and McNeilly, 1991; McNelly, 1991) and by supplying substrate for aromatization in follicular phase.

As the preovulatory period progresses, the basal level of LH is capable of causing androgen production from theca cells and inducing LH receptors on granulosa cells, thereby causing the secretion of oestradiol that is not dependant on FSH (Webb and England, 1982; Baird, 1983). The secretion of oestradiol from the preovulatory sheep follicle depends on secretion of LH.

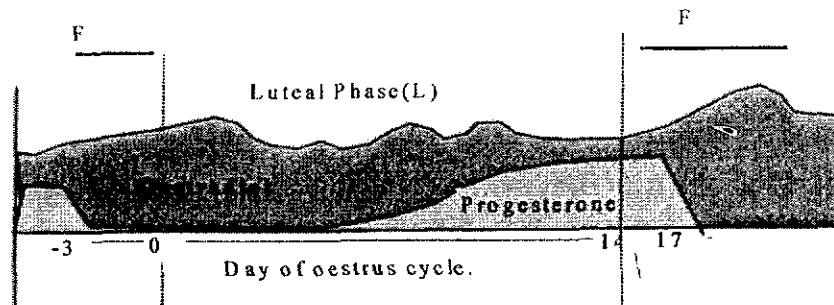
2.1. 1. 2. THE INTERACTION BETWEEN PITUITARY GONADOTROPHINS AND OVARIAN STEROIDS AND THEIR FUNCTION IN THE SELECTION OF DOMINANT FOLLICLE.

The selected follicle is the follicle that can survive as the FSH concentration falls and the LH pulse frequency rises during the follicular phase (McNeilly *et al.*, 1992). This follicle has entered the gonadotrophin-dependent phase and produces cAMP and oestradiol in response to gonadotrophins. Selection occurs when the follicles become gonadotrophin-dependent. In sheep, non-atretic follicles bigger than 2.5mm in diameter are usually considered gonadotrophin-dependent (Scaramuzzi *et al.*, 1993; Fortune *et al.*, 1991). Follicles bigger than 4mm can be identified as ovulatory follicles which have the receptors for both FSH and LH in granulosa cells (Fortune *et al.*, 1991).

During selection, oestradiol and inhibin suppress FSH to below the concentration required for the development of subordinate follicles and causes these follicles to become atretic. Thus, a reduction of FSH during follicular phase can alone be responsible for selection of ovulatory follicles.

Follicular growth and differentiation take place under the influence of both gonadotrophins and ovarian steroids especially oestradiol. Oestradiol is formed in granulosa cells by aromatisation of androgen substrate produced in theca cells under the control of LH. FSH is required to induce the aromatase system. Most of the oestrogen in plasma is derived from large preovulatory follicles. Oestrogen synthesis from these follicles can be enhanced by autocrine and paracrine actions of oestrogens and other intraovarian factors, such as cytokines and by the appearance of the LH receptors on granulosa cells. Oestrogen appears to protect the growing follicle from androgen-induced atresia because atretic follicles are characterised by a low follicular fluid oestrogen concentration and a decrease in the oestrogen/androgen ratio (Marko Filicori, 1999).

Oestradiol in serum increases on day 3 of the luteal phase. This increase in oestradiol is a response to increased FSH secretion on the first day of the oestrus cycle (Figure; 2. 1. 1. 2. 1). After this increase, the FSH concentration decreases and remains almost at the same level until day six. On day six of the cycle, FSH concentrations increase again.



Figure; 2. 1. 1. 2. 1; Changes in the concentration of the ewe of oestradiol and progesterone in peripheral blood during the oestrus cycle. The oestrus cycle of ewe is divided into luteal and follicular phases. The luteal phase starts on day 2 and continues until day 13. During the luteal phase, a new corpus luteum becomes active and produces progesterone, which begins to increase around day 3-4 of the oestrus cycle. Luteolysis starts on day 14 of oestrus cycle. At luteolysis, the corpus luteum begins to regress and the concentration of progesterone in blood decreases and reaches its lowest value within 24 hours. At luteolysis, the concentration of oestradiol begins to increase in response to increased LH pulse frequency. As the oestradiol concentration begins to increase, the follicular phase starts. Therefore the follicular phase starts on the day 14 and ends on days 3-4 of oestrus cycle (Adapted from fig. 1. of Scaramuzzi *et al.*, 1993).

Progesterone secretion decreases at luteal regression and this is followed by a rise in the concentration of LH and oestradiol. LH secretion during the luteal phase is suppressed by progesterone and increased LH secretion after luteal regression stimulates the secretion of oestradiol by increasing the secretion of thecal androgen. Therefore, LH plays an indirect role in follicle selection by suppressing the effect of FSH and increasing the rate of atresia (Baird, 1983).

Oestradiol alone can enhance the responsiveness of granulosa cells to FSH by increasing number of FSH receptors in hamster and rat (Harman, 1975; Wang and Greenwald, 1993). Oestradiol and FSH together induce receptors for LH and FSH in both theca and granulosa in all species respectively. In the rat, the number of these receptors is high during follicular development and low during atresia and early

lutinisation (Kamiyashi *et al.*, 1986; Sar and Welsch 1999). FSH binding to its receptor stimulates cAMP production, which, in turns increases expression of mRNA for LH receptors (Richards *et al.*, 1977).

2.1.1.3. ROLE OF PITUITARY GONADOTROPHINS AND OVARIAN STEROIDS IN FOLLICLE MATURATION

The feedback mechanism between gonadotrophins and ovarian steroids (androgens, oestrogens and progestins) regulates follicle maturity. A mature ovarian follicle has receptors for LH and FSH and has maximum responses to gonadotrophins and produces high amounts of oestradiol. Thus, oestradiol secretion is a marker of follicle maturity (Lacker, 1981), Oestradiol also controls the secretion of gonadotrophins.

FSH serves as the primary inducer of granulosa cell maturation. It acts together with oestradiol to activate granulosa cell proliferation, enhance aromatase enzyme activity and the expression of LH receptors on theca and granulosa cell. Theca cells always express LH receptors, whereas granulosa cells only express LH receptors in the late follicular phase. Receptors for FSH are expressed on granulosa cells from early preantral follicles. Since recruited and selected follicles express only FSH receptors on their granulosa cells they are very susceptible to falls in FSH concentrations, and readily become atretic (Zelevnik and Hillier, 1996; Cortvrindt *et al.*, 1998).

LH acts on the theca to provide androgen substrate for the aromatase enzyme system to produce oestrogen (Filicori, 1999). Although LH delivery is pulsatile this may not be essential (McNatty *et al.*, 1981).

2.1.2. THE AUTOCRINE AND PARACRINE ROLE OF GROWTH FACTORS IN FOLLICULAR GROWTH AND DIFFERENTIATION.

If a cell secretes an agent which has an effect on the same cell, is an autocrine effect; if a secreted agent exerts its effect on an adjacent cell, it is a paracrine effect and If a secreted agent is carried by blood circulation to a distant target site of action, it is an endocrine effect (Figure 2.1.2.1.) (Eckert *et al.*, 1988). Growth factors have been classified into separate families according to their structure and biological activity; the epidermal growth factor family (EGF), the fibroblast growth factor family (FGF), the

insulin-like growth factor family (IGF), the transforming growth factor family- β (TGF- β), the platelet derived growth factor family (PDGF) and the haemopoietic growth factor (cytokines) family (Mondschein and Schomberg, 1981). Each family of growth factors include at least three elements; the growth factor, their signalling receptors and their binding proteins (Monget and Monniaux, 1995). Most growth factors enhance the action of gonadotrophins by modulating hormone-dependent differentiation (Cell proliferation, steroidogenesis, gonadotrophin sensitivity, cytoplasmic and nuclear maturation) in endocrine target cells (Roy and Greenwald, 1991; Skinner and Parrott, 1994).

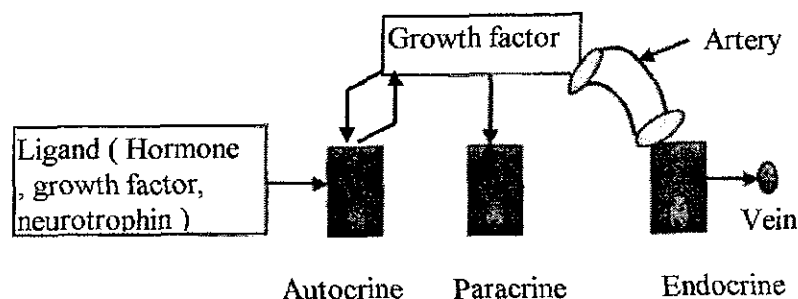


Figure 2.1.2.1.; The concept of autocrine paracrine and endocrine secretion (Adopted from Ericson and Danforth, 1995). Hormones act in many different ways (autocrine, paracrine and endocrine) to regulate cellular function. Growth factors generally (except IGF-1, relaxin and inhibin) do not act as endocrine factors, but diffuse from their site of production through intercellular spaces to influence neighbouring cells that contain appropriate receptors (Dorrington *et al.*, 1987).

For instance, the rat granulosa cells secrete insulin-like growth factor-1 (IGF-1) under the action of growth hormone (Davoren and Hsueh, 1986). This IGF-1 has an autocrine effect on granulosa cells, stimulating the tropic action of FSH resulting with FSH dependent LH receptor induction, enhanced aromatase activity and the production of oestradiol and inhibin. It also acts in a paracrine way to stimulate androgen production from the theca cells (Carson *et al.*, 1989).

2.1.2.1. GROWTH FACTORS AND FOLLICULAR FUNCTION.

Growth factors modulate follicular growth and differentiation by modulating the actions of gonadotrophins (Dorrington *et al.*, 1987). They vary in their effects on gonadotrophin-induced follicular differentiation (Erickson and Danforth 1995). They exert their effect using a variety of possible mechanisms including direct mitotic action, modulation of steroidogenesis, gonadotrophin receptor induction and signal transduction (Table 2.1.2.1).

Table 2.1.2.1. Effects of growth factors on follicular function

Factor	Mouse	Rat	Sheep
EGF	Inhibits mitosis of follicular cells, FSH-induced secretion of oestradiol and secretion of progesterone from granulosa	Decreases FSH-dependent LHR induction, aromatase activity, oestradiol, inhibin production from granulosa cells, cAMP formation preovulatory follicular growth.	Inhibits or stimulate mitosis of follicular cells in dose dependent manner, Inhibits oestradiol production, increases progesterone production
IGF	Increases FSH-induced mitosis, aromatase activity and oestradiol production from granulosa and FSHR induction on granulosa	Increases FSH-dependent LHR induction, aromatase activity, oestradiol, inhibin production from granulosa cells and androgen production from theca.	Increases mitosis of granulosa cells, FSH-induced LHR induction aromatase activity, oestradiol secretion from granulosa
Inhibin & activin	Regulates secretion of FSH. Activin stimulates FSH-induced follicle growth, oestradiol, inhibin production from granulosa.	Regulates secretion of FSH and gonadotrophin-induced steroid and cAMP production. Inhibin enhances follicular recruitment. Activin promotes atresia and blocks FSH-induced follicular development	Inhibin decreases FSH secretion, oestradiol production, follicular growth. Increases follicular fluid concentration of activin A.

Continued from table 2.1.2.1.

TGF-α	Decreases preovulatory follicle growth, progesterone, oestradiol production from granulosa.	Inhibits FSH-induced oestradiol production, increases pregnenolone, 20 α -HSD activity, progesterone production from granulosa cells <i>in vitro</i> .	Decreases oestradiol and inhibin production, induces atresia in large antral follicles <i>in vivo</i> . Stimulates mitosis of theca and granulosa in dose dependent manner.
TGF-β	Increases FSH-induced mitosis of follicular cells and FSH-induced oestradiol and progesterone production from granulosa	Decreases FSH-induced LHR induction but it increases FSHR mRNA expression, increases FSH-induced aromatase activity and oestradiol secretion from granulosa cells <i>in vitro</i> . Increases induction of FSH-induced EGF-receptors on granulosa cells	No data available
GDF-9	Facilitates follicle growth, Increases FSH-induced LHR induction, inhibin production, P450arom activity in granulosa,	Increases mitosis of granulosa cells, preantral follicle growth, suppresses FSH-induced oestradiol and progesterone production	Regulates early follicular growth
PDGF	No data available	Increases FSH-induced LHR induction, cAMP and progesterone production from granulosa	No data available
FGF	No data available	bFGF and IGF-1 increases expression of LHR on granulosa cells in dose dependent manner. Together with IGF-1 enhances granulosa cell production of cAMP and prostaglandin E2 in culture.	Increases mitosis of granulosa cells, together with IGF-1 induces LHR induction on granulosa cells, decreases oestradiol and androstenedione production

They act with FSH and none of them alone appear capable of effecting granulosa cells. Granulosa cells contain receptors for most growth factors (Tonetta and diZerega, 1989; Roy 1993) and the binding activity of growth factors often facilitated by FSH. For instance; during pro-oestrus and oestrus, there is a greater amount of FSH circulating in the blood which increases the binding activity of EGF to its receptors on granulosa cells (Fujinaga 1994). EGF facilitates progesterone production and therefore, increases the rate of atresia (Murray *et al.*, 1992). It has similar effects, in the presence of FSH as Transforming Growth Factor- α (TGF α) which increases the production of progesterone from rat granulosa cells *in vitro* (Yeh *et al.*, 1993). This increase in progesterone production is probably due to increased pregnenolone biosynthesis and 20 α -HSD activity. It has no stimulatory effect on androgen production (Modschein and Schomberg, 1981; Jones *et al.*, 1983; Yeh *et al.*, 1993).

Epidermal growth factor (EGF) has opposite actions to those of insulin and Insulin-like growth factor-1 (IGF-1) on FSH-induced aromatase activity, but it acts in the same manner on IGF-1 to increase mitotic activity of granulosa cells (Dorrington *et al.*, 1987b).

Insulin Like Growth Factor-1 (IGF-1) has been identified as follicle survival factor (Erickson and Danforth, 1995; Haanen and Vermes, 1996). Insulin like Growth Factor-1 activates FSH-induced adenylate cyclase activity, cAMP production and LH receptor formation in granulosa cells. It stimulates P450_{arom} enzyme activity in granulosa and P450_{scc} enzyme activity in theca resulting in increased secretion of oestradiol, inhibin, androgen and progesterone. Insulin Like Growth Factor-1 does not effect the basal levels of oestradiol and testosterone, but it stimulates pregnolone and progesterone production by increasing P450_{scc} enzyme activity in mitochondria (Giudice 1992). Increased progesterone synthesis by IGF-1 and FSH is enhanced by oestradiol-17 β , which enhances high and low density lipoprotein metabolism resulting in increased amounts of androgen.

IGF-1 and EGF potentiate mitosis of granulosa cells in small follicles. Insulin like Growth Factor-1 is more potent than EGF in stimulating the incorporation of [³H] thymidine into the DNA of granulosa cells in small follicle (Dorrington *et al.*, 1987b). After antrum formation, the effect of IGF-1 becomes much more important for follicular cell differentiation. Epidermal Growth Factor inhibits FSH-induced aromatase activity of granulosa cells, but insulin and IGF-1 stimulate FSH-induced aromatase activity (Dorrington *et al.*, 1987b).

Inhibin, activin and Transforming Growth Factor- β , are products of the same gene family and they have both positive and negative effect on FSH-dependent granulosa cell function and LH-dependent thecal cell function. When follicle diameter increases and the follicle approaches maturity inhibin production increases and contributes to continued follicle growth and perhaps follicle selection via stimulation of thecal androgen production. In immature follicles granulosa cell production of activin is higher than inhibin and activin stimulates folliculogenesis via the acquisition of granulosa cell aromatase activity and FSH receptor induction. Inhibin acts synergistically with LH and IGF-1 to increase androgen production by theca cells in culture (Giudice 1992; Symth *et al.*, 1993) but, activin inhibits theca androgen

production by rat granulosa cells (Findlay 1993). Injection of inhibin into the intrabursal space of rats results in increased follicle growth, whereas injection of activin leads to increased atresia.

Transforming growth factor- β alone has little positive effect on epidermal growth factor receptor formation, but it facilitates FSH-induced epidermal growth factor receptor formation on rat granulosa cells (Feng *et al.*, 1986).

Recently a new member of TGF- β family (GDF-9) has been identified in the oocyte of mice in all stages of follicular development (McGrath *et al.*, 1995; Dong *et al.*, 1996). In GDF-9 knockout female mice, primordial and primary one-layer follicles were formed, but there was a block in follicular growth beyond the primary stage and these females were not fertile (Dong *et al.*, 1996). In hamster granulosa cell culture, when the rGDF-9 was not supplemented, expression of mRNA's for LHR and P450 α were reduced and secretion of progesterone increased (Elvin *et al.*, 1999; Hayashi *et al.*, 1999). Growth and Differentiation Factor-9 is required for follicular growth it enhanced the growth of rat follicles in culture (Elvin *et al.*, 1999).

Platelet-Derived Growth Factor (PDGF) increases FSH-induced LH receptor induction on granulosa cells from rat (Mondschein and Schomberg, 1981). It enhances cAMP production and progesterone production in granulosa cells (Carson *et al.*, 1989).

2.1.2.2. THE ROLE OF TGF- β IN GONADOTROPHIN RECEPTOR INDUCTION ON GRANULOSA CELLS AND THE REGULATION OF OVARIAN STEROID SECRETION.

Transforming Growth Factor- β is produced by granulosa and theca within the ovary. However, granulosa and theca cells appear to be sites of TGF β production but, there is a fundamental difference in the regulation of TGF β secretion by the two cell types (May *et al.*, 1996). Secretion from the theca involves the activation of a protein kinase-C pathway and not the cAMP/protein kinase A pathway, whereas activation of neither of these pathways appears to regulate inducible TGF β secretion in granulosa cells (May *et al.*, 1995). This hormonally active polypeptide consists of two 12kDa chains linked by disulphide bonds to form a 23-25kDa dimer (Massague, 1985).

Transforming growth factor- β has autocrine actions on granulosa and theca cells resulting in the modulation of theca and granulosa cell steroidogenesis and gonadotrophin receptor induction. The effect of transforming growth factor- β on gonadotrophine receptor induction depends on the species. Transforming growth factor- β alone increased LH receptor formation on pig granulosa cells, whereas it decreased LH receptor formation on rat granulosa cells during 3-6 days of culture (Gitay-Goren *et al.*, 1993). Addition of transforming growth factor- β , into pig thecal cell culture, caused luteinization of the cells and the production of androgen (Engelhardt *et al.*, 1992). High or low doses of transforming growth factor- β decreased LH induced progesterone secretion from pig thecal cell *in vitro* without any effect on the activity of cholesterol sidechain cleavage and 3 β -hydroxysteroid dehydrogenase enzymes.

Transforming growth factor- β facilitated the effect of low concentration of FSH on LH receptor formation, whereas it inhibited the effect of high doses of FSH on LH receptor formation. *In vitro* experiments in rats, showed that treatment of granulosa cells for 48h with TGF β 1 increased the level of FSH receptor mRNA (Dunkel *et al.*, 1994). There is a close parallelism between FSH receptor mRNA levels and FSH receptor content (Gitay-Goren *et al.*, 1993; Dunkel *et al.*, 1994). Changes in FSH receptor number is associated with similar changes in the levels of 7 and 2.5 kb mRNA transcripts for the FSH receptor. Transforming Growth Factor- β 's are involved in the differentiation of granulosa cells by modulating FSH receptor gene expression and FSH-stimulated LH receptor numbers. The formation of FSH and LH receptors are important for granulosa cell differentiation (Dunkel *et al.*, 1994).

Receptors for TGF β are present in granulosa cells of primordial and preantral follicles (Levacher *et al.*, 1996). The receptors for TGF- β_2 and β_3 , are both Serine/Threonine kinases. The signalling mechanism induced through these receptors is not well understood.

TGF- β increased FSH induced aromatase activity and oestradiol secretion from rat granulosa cells in culture (Dorrington *et al.*, 1993). Interactions between oestradiol, FSH and TGF β led to rapid increase in serum oestradiol levels during the follicular phase in rats, but it had no *in vitro* effect on basal progesterone synthesis at any stage of follicular development (Lafrance *et al.*, 1993).

2.1.2.3. THE TGF- β RECEPTOR SUPERFAMILY: LIGAND BINDING AND CELL SIGNALING.

Transforming Growth Factor- β mediates its action by binding to cell surface receptors. The receptors for this family of growth factors consist of a small exterior domain ending at the amino terminal of the protein, a single transmembrane domain and a large cytosolic domain at the carboxyl terminal (Figure 2.1.2.5.1.).

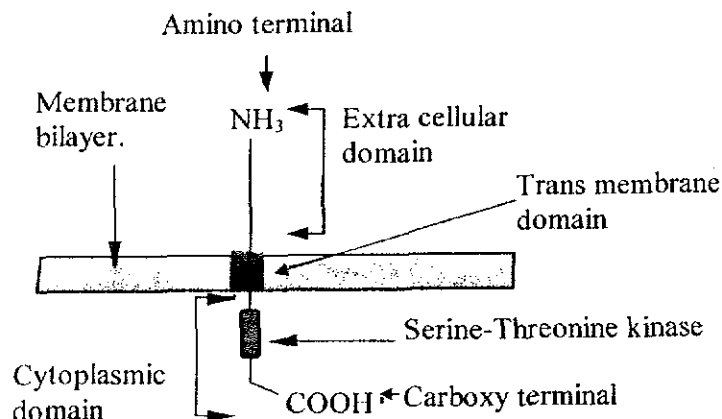


Figure 2.1.2.3.1.; The unoccupied TGF- β receptor has no tyrosine kinase activity (Lodish *et al.*, 1995). Activation of receptor by ligand binding induces Serine-Threonine phosphorylation. The receptor consists of three parts; a small external domain, a single pass transmembrane domain and a large intracellular domain that contains a Serine-Threonine kinase.

Receptors for this growth factor are ligand-activated protein kinases. Ligand binding leads dimerization of the receptor and activation of the Serine-Threonine kinase. A single mammalian cell may contain more than 100 distinct kinds of kinases many of which are Serine-Threonine kinases.

The mechanism by which receptor Serine-Threonine kinases transduce signals is poorly understood. Protein kinases covalently add phosphate groups to the signalling proteins, these proteins in turn, generally cause the phosphorylation of downstream proteins. The phosphorylation sites of Serine and Threonine amino acids are shown in figure 2.1.2.3.3. Cell survival and or proliferation are generally stimulated by specific combinations of signals rather than by a single signal. In figure 2.1.2.3.2. signals X and

Y activate different cascades of protein phosphorylation each of which leads to phosphorylation of the same protein (A), but at different sites. Protein A is activated only when both sites are phosphorylated. Therefore activity of protein A is active only when signals X and Y are simultaneously present.

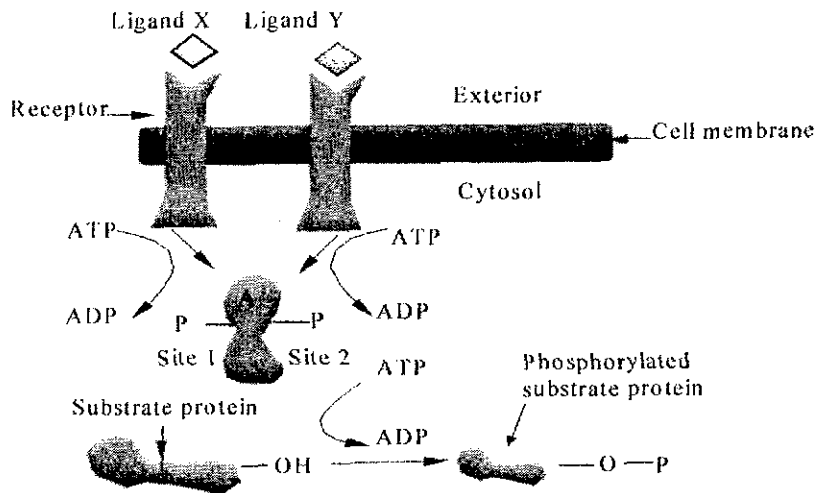


Figure 2.1.2.3.2; Protein A is only active when both sites are phosphorylated by Serine-Threonine kinases and it is not yet known if Serine-Threonine kinases phosphorylate specific substrate proteins in the cell (For more information see above (Modified from Alberts *et al.*, 1994 and Lodish *et al.*, 1995)).

The phosphorylation of Serine-Threonine residues in substrate proteins may stimulate new-high affinity binding sites for other intracellular signalling proteins serving as a key to initiate downstream signalling.

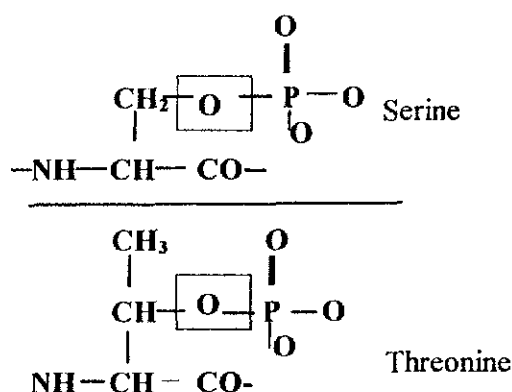


Figure 2.1.2.3.3.; The phosphorylation sites of Serine and Threonine.

Cellular function is also regulated by the phosphorylation of Serine / Threonine in eukaryotic cells in response to external stimuli. Many cellular functions such as transcription, translation, ion transport, cell re-modelling, mitotic activity and the cell cycle are regulated by phosphorylation of Serine/Threonine in target proteins. Examples include the cAMP-responsive enhancer element binding (CREB) protein and the cAMP-responsive enhancer element modulator (CREM). These proteins are nuclear transcription factors and phosphorylation of CREB on Serine 133 is important for gene transcription. These proteins regulate the expression of mRNA for TGF- β like peptides (Potchinsky *et al.*, 1997).

2.1.2.4. THE IGF SYSTEM AND ITS EFFECT ON FOLLICULAR CELL PROLIFERATION AND OVARIAN STEROID SECRETION.

The IGF system consist of three different parts, two ligands (IGF-1,IGF-2 and IGF-3) which are structurally related to insulin, two receptors and six binding proteins (Figure 2.1.2.6.1). Insulin like Growth Factor-1 and 2 are complexed with the family of binding proteins (IGFBP's) in serum, amniotic and other body fluids. These binding

proteins modulate the autocrine and paracrine action of the ligands. The binding proteins inhibit the mitogenic effect of IGFs (IGF1 and IGF-2) by limiting peptide access to specific cell surface receptors (Giudice, 1992).

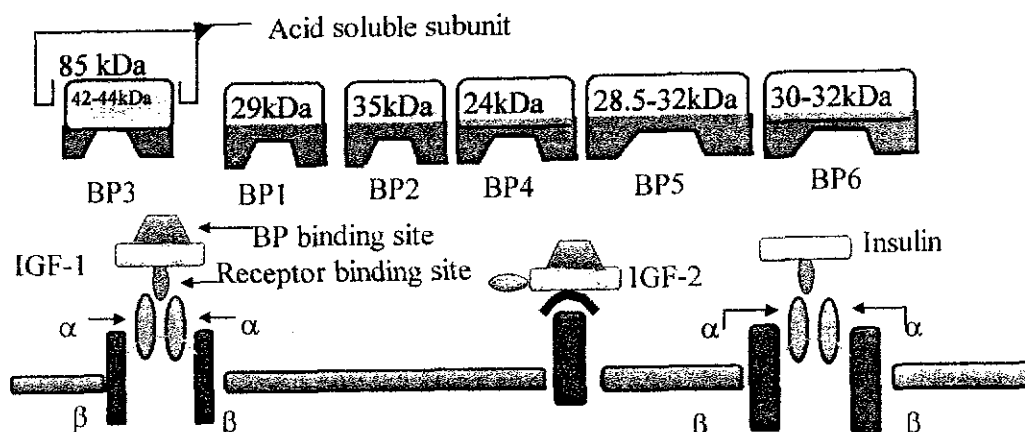


Figure 2.1.2.4.1; The IGF system is consist of IGF-1 and IGF-2, two receptors (The IGF-1 receptor and the IGF-2 or Mannose-6 Phosphate receptor) both of which are present at high levels in the granulosa of atretic follicles and in theca of healthy follicles. There are six binding proteins in IGF system (BP1, BP2, BP3, BP4, BP5 and BP6). Binding proteins are believed to prolong the half-life of the ligands by protecting them from degradation. Concentrations of binding protein of low molecular weight (IGBP-4 and 5) are increased in granulosa during atresia (Modified from figure 1 of Monget and Monniaux, 1995).

Insulin like Growth Factor-1 is a 130 amino acid protein. Its secretion is dependent on growth hormone. Previously, liver had been shown as a site of IGF-1 synthesis but, recently, it has been suggested that it is also synthesised by extrahepatic tissues where it may play autocrine and or paracrine regulatory roles (Hammond *et al.*, 1991).

The rat ovary is a site of IGF-1 secretion and action. Insulin like Growth Factor-1 receptors have been identified and characterised in the sheep ovary and these receptors (Type 1) mediate most of the effects of IGF-1 on theca and granulosa cells (Teissier *et al.*, 1994). The type one receptor has a molecular weight of 350 kDa and appears to be an integral membrane glycoprotein similar to the insulin receptor. The

type one receptor is present on sheep granulosa cells (Monget *et al.*, 1989). This receptor consist of two subunits, α and β . These two units are joined by interchain disulphide bridges. The α subunit is believed to occupy the extracellular part of the receptor forming the ligand binding site and the β subunit occupies the intra cellular part of the receptor and displays IGF-1-dependent tyrosine kinase activity capable of aoutophosphorylation. The expression of these receptors in granulosa cells is increased by oestrogen and gonadotrophins in rat and swine (Adashi *et al.*, 1986; Veldhuis *et al.*, 1986).

In sheep, IGF-1 stimulates mainly proliferation of granulosa cells from small follicles (1-3mm), whereas it simulates secretion of progesterone by granulosa cells from large (>5mm) follicles (Hammond *et al.*, 1991; Monniaux and Pisselet, 1992; Monget and Monniaux, 1995). Insulin like Growth Factor-1 by itself has a limited effect on granulosa cell progesterone biosynthesis and the degree of this effect depends on time and the dose employed (Monniaux and Pisselet, 1992). Progesterone secretion, from granulosa cells, is not dependend on FSH (Adashi *et al.*, 1985). Insulin like Growth Factor-1 increases FSH-induced granulosa cells aromatase activity, LH receptor formation and oestradiol production (Adashi *et al.*, 1985).

Insulin like Growth Factor-2 is a 180 amino acid protein, it is only partially growth hormone dependent. Receptors for IGF-2 consistently bind IGF-2 with higher affinity than IGF-1 and have very little or no affinity for the insulin. It is synthesised by various tissues including the gonads. Insulin like Growth Factor-2 has mitogenic effect on follicular cells from human (Di Blasio *et al.*, 1994).

2.1.2.5. FIBROBLAST GROWTH FACTORS (FGFs) AND THEIR ROLE IN FOLLICULAR GROWTH AND STERIDOGENESIS.

Fibroblast growth factors are present in ovaries in two closely related forms, basic and acidic (bFGF and aFGF) (Gospodarowicz and Ferrara, 1989). Basic Fibroblast Growth Factor is a single chain polypeptide composed of 146 amino acids and it has an isoelectric point of 9.6. Acidic Fibroblast Growth Factor composed of 140 amino acids and it has an isoelectric point of 5.6-6 (Gospodarowicz *et al.*, 1986). Basic, but not acidic fibroblast growth factors (FGF), from follicular cells, can act in an autocrine way to stimulate proliferation of follicular cells (Neufeld *et al.*, 1987). It

has been shown to inhibit both FSH-induced LH receptor expression and aromatase activity in rat granulosa cells (Baird and Hsueh 1986; Oury and Darbon 1988). It also causes a dose dependent decrease in oestradiol secretion by undifferentiated rat granulosa cells (Adashi 1988). The extracellular localisation of bFGF is variable. Its localisation is altered during follicular atresia. In atretic follicles, there is an enhanced degree of localisation within the antral fluid. This localisation seems to stimulate growth of fibroblast like cells within the antral cavity during atresia (Wordinger *et al.*, 1993).

Sheep granulosa cells produce bFGF (BK Campbell and RJ Scaramuzzi, unpublished observation; Reynolds and Redmer 1998) and it is mitogenic for granulosa cells maintained *in vitro* (Grazul-Bilska *et al.*, 1992). The mitogenic and morphogenic activity of bFGF is regulated (positively or negatively) by thecal TGF β (Gospodarowicz *et al.*, 1977; Gospodarowicz and Ferrara, 1989). It is a potent inhibitor of maturation of immature granulosa cells and it inhibits pulsatile secretion of oestradiol and androstenedione (Vernon and Spicer, 1994; Scaramuzzi and Downing, 1995). Basic Fibroblast Growth Factor together with IGF-1 induces LH receptors on rat granulosa cells by mechanisms involving production of prostaglandin E2 (Kanzaki *et al.*, 1994).

Infusion of ewes with autotransplanted ovaries for 12h with 1.5 μ g/hour with bFGF suppressed the secretion of oestradiol and androstenedione during, and for up to 30h after the infusion but, the infusion of FGF had no detectable effect on the secretion of progesterone (Scaramuzzi and Downing, 1995). This reversible action of bFGF is time and dose dependent. It seems that decreased oestradiol secretion is a result of decreased androgen precursor for aromatisation (Hurwitz *et al.*, 1990).

2.1.2.6. EPIDERMAL GROWTH FACTOR (EGF) AND ITS EFFECT ON FOLLICULAR CELL PROLIFERATION AND DIFFERENTIATION

Epidermal Growth Factor is a single chain polypeptide and its effect on follicle growth and steroidogenesis varies according to species (Boland and Gosden, 1994). In the rat and mouse, it inhibits mitosis of follicular cells, but in sheep, it does not prevent follicle development to preovulatory size (Almahbobi *et al.*, 1995). It has no mitogenic effect in sheep (Shaw *et al.*, 1985; Fujinaga *et al.*, 1992). In the hamster, EGF

increases FSH-induced DNA synthesis, and this effect of EGF is inhibited by TGF β (Roy, 1993).

The effect of EGF on the follicle is dose dependent. Higher doses of EGF inhibit adenylate cyclase activity resulting in the inhibition of cAMP formation and FSH-induced LH receptor induction on rat granulosa cells without preventing preovulatory follicular growth both *in-vivo* and *in-vitro* (Modschein and Schomberg, 1981; Jones *et al.*, 1983; Schomberg *et al.*, 1983; Boland and Gosden 1994). The effect of EGF on follicular differentiation also varies according to the species. For instance, in rat granulosa cells, it inhibits FSH-induced LH receptor formation, aromatisation and oestradiol production, whereas it causes luteinization of hamster granulosa cells and stimulates progesterone and 20 α -dehydroprogesterone production (Schomberg *et al.*, 1983; Fujinaga *et al.*, 1992).

Infusion of ewes with EGF in the luteal phase had no detectable effect on progesterone synthesis, but it inhibited oestradiol production resulting in the suppression of oestrus. Infusion of EGF in the follicular phase, at concentrations greater than 9ng/ml, completely inhibited oestradiol production and enhanced progesterone production, but the infusion had no effect on androstenedione production (Murray *et al.*, 1993). This treatment increased secretion of FSH and LH because of the disruption of inhibin and oestradiol negative feedback on the hypothalamo-pituitary axis (Murray *et al.*, 1993).

Increased progesterone production is probably due to increased *de novo* synthesis of cholesterol (Shaw *et al.*, 1985). Increased progesterone production is a result of the atretic effect of EGF on follicles due to the increase in P450_{scc} enzyme activity within the mitochondria (Magoffin *et al.*, 1989). Atretic follicles, corpus lutea and theca lutein cells contain large amounts of EGF (Wei *et al.*, 1997).

2.2. FOLLICULOGENESIS AND THE OESTROUS CYCLE IN MICE

The mouse is a polyoestrous animal. The length of the oestrous cycle is 4-5 days (Campbell *et al.*, 1976). The duration of heat is 9-20h and 2-3h after the onset of oestrus, ovulation occurs (Hafez 1980). Ovulation, in mice, occurs spontaneously. The first oestrous is seen at 31-39 days of age and the first ovulation, occurs at approximately 35-45 days of age and vaginal opening occurs around day 38 after

birth. Ovulation may not occur at every oestrus, particularly in young virgin females. The period between birth and ovulation depends on the presence of males and light rhythms. It was reported that sexual maturation of female mice is accelerated by the presence of an intact male (Vandenbergh 1969, Grasso *et al.*, 1997). Mice are more resistant to total disruption of oestrus by constant light, compared with the rat, despite the fact that their steroid patterns are altered. In the rat, lighting plays a dominant role in the incidence and duration of the oestrous cycle (Campbell *et al.*, 1976, Freeman, 1994).

During the oestrous cycle, the cellular contents of the vagina undergo cyclical changes. Oestrus is characterised by squamification and cornification of the cells and the disappearance of leukocytes. Therefore, the oestrous cycle of mouse is conventionally divided into five stages namely proestrous, oestrous, metestrous-1, metestrous-2 and diestrous (Snell 1941, Hafez, 1980). The first two stages of oestrous cycle are anabolic and during this stage active growth of different parts of the genital tract is in progress. The other two stages (metestrous-1, metestrous-2) of the oestrous cycle are catabolic and characterised by degenerative changes in the genital tract. The last, diestrous is a period of quiescence or slow growth of the genital tract (Snell 1941). These different stages of oestrous cycle are characterised by the smear technique which involves the identification of the cell types and their relative quantities present in preparations obtained from the vaginal wall (Snell 1941, Chaplin *et al.*, 1973). By these methods three types of cells are identified: Leukocytes, cornified cells and nucleated epithelial cells. The complete or almost complete absence of leukocytes from the smear and the presence of cornified cells, in moderate numbers are the usual criteria of oestrous (Hafez, 1980).

Ovarian cycle continues throughout the year, and ovulation occurs every 4-5 days. The ovary of unborn mice contains primordial germ cells, by 12 day of gestation they are at meiotic prophase and all of them reach at the resting stage by birth. From an oogonium to a fully developed Graafian follicle takes 30-34 days. The period from primordial stage to Graafian stage takes approximately 14-16 days. The ovary of 21 days old prepubertal mice contain fully developed follicles (Grasso *et al.*, 1997). Neonatal follicular development is under the influence of FSH. A massive and prolonged peak of serum FSH is present in unborn female mice (Grasso *et al.*, 1997), and administration of antibodies to FSH arrests neonatal follicular development.

Pituitary gonadotrophins not only regulate the growth of medium and large sized follicles but also control the recruitment of primordial follicles into the growing pool (Wang and Greenwald, 1993). Both preantral and antral follicles in the mouse appear to be regulated by gonadotrophins. The effect of hypophysectomy on folliculogenesis was investigated by Wang and Greenwald (1993) and they reported that the number of follicles at all stages, including the smallest growing follicles decreased significantly after 4 days hypophysectomy.

The preovulatory period of the oestrous, in mice, is characterised by a growth of ovarian follicles and a concomitant enhanced secretion of oestrogen. The secretion of oestrogen is low on oestrous, begins to rise significantly by late metestrous through the morning of diestrous and reaches the peak concentration by the afternoon of proestrous. The secretion of testosterone and androstenedione, during the oestrus, are similar in pattern of oestradiol.

Antrum formation first occurs in follicles about 200 μ m in diameter. Follicles which will ovulate at the following oestrous are on average 380 μ m in diameter at the beginning of the oestrous cycle. The follicles reach a maximum size of an average of 550 μ m immediately before rearing. Most of the growth occurs in the last 48h.

Progesterone is required for maintenance of pregnancy in the mouse (Hafez, 1980). Progesterone and 20 α -hydroxyprogesterone are secreted by the newly formed corpus luteum from metestrus through diestrus. The secretion of progestin declines throughout the diestrus and a new ovulation takes place shortly after. During the oestrous cycle, 3 or more generations of corpus luteum may be present on the ovary from the immediately preceding ovulatory cycles. By diestrus, the newly formed corpus luteum have attained their maximal size which is maintained through metestrous of following cycle and the corpus luteum regress by diestrus. During diestrus, secretion of progestin declines and a new ovulation takes place shortly after (Freeman, 1994). In rodents, stimulation of uterine walls or unfertile mating causes the secretion of luteotrophin from pituitary gland which is sufficient to rescue corpus luteum and allow it to persist for a number of days (about 12-14 days). This period is known as pseudupregnancy (Freeman 1994). If the mating is fertile, the corpus luteum persists for the entire period of pregnancy (about 19 days). This luteotrophic agent, causing pregnancy, has been reported to be prolactin secreted from anterior pituitary gland.

Prolactin suppresses the activity of 20 α -hydroxysteroid dehydrogenase which converts progesterone to 20 α -hydroxyprogesterone which is not effective for the maintenance of pregnancy. Therefore, prolactin maintains progesterone secretion.

Male mouse become sexually mature by 40 day after the birth. Time of sexual maturation is effected by the presence of female mice. The testis of male mice is not well developed as compared with mice grown in the presence of females. The presence of females facilitates the secretion of testosterone and this in turn causes full development of the accessory sex organs (Jean-Faucher *et al* 1984). Absence of females prolongs the period of sexual maturation.

2. 3. NUTRITION AND REPRODUCTION

2. 3. 1. EFFECTS OF NUTRITION ON REPRODUCTION

The reproductive ability of an animal is influenced by its genotype, age, time of the year and nutrition (Rhind, 1992). Increased ovulation rate of lupin fed ewes is the result of the high energy and protein content of lupin grain (Teleni *et al.*, 1984 and Downing *et al.*, 1995a). Lupin feeding increases the amount of energy yielding substrates (such as glucose) in the blood (Freetly and Klindt, 1996). A short period (3 hours) of infusion of sheep with glucose or acetate that provided equal amounts of energy as supplementation with lupins also increased ovulation rate (Teleni *et al.*, 1989). Long periods of infusion of energy yielding substrates also increased LH pulse frequency of rams 5-10 days after infusion, but the increase was smaller than when the sheep received lupin grain (Boukhliq, 1997). Whereas, Downing *et al.*, (1995) reported that the intravenous infusion of glucose for 5 days, during the luteal phase, had no significant effect on LH pulse frequency. Increases in ovulation rate in lupin fed ewes may not be entirely the result of the high energy and protein content of lupins, there may be an effect of a specific component of lupins (Blache *et al.*, 1996). For normal function, brain utilises glucose. In *in vitro* condition, brain tissue is remarkably resistant to low glucose level, but when glucose level declines to 2mM *in vivo* unconsciousness occurs (Bradford, 1986). It was postulated that, glucose participates directly in critical neuronal function rather than neurotransmitter synthesis (Bradford, 1986). Glucose is metabolised to excitatory amino acids (such as glutamate, aspartate,

gamma-aminobutyric acid (GABA) glutamine and alanine) within the brain (Bradford, 1986; P'Anson *et al.*, 1991). Studies by using [U-¹⁴C] confirmed that; the carbon skeletons of the dicarboxylic acids (glutamate and aspartate) are rapidly formed from glucose. Glutamate and aspartate formed by transamination of 2-oxaloacetate formed in the citric acid cycle. Gamma-aminobutyric acid is formed by decarboxylation of glutamate by decarboxylase (Bradford, 1986). These amino acids have neuroendocrine effects on the hypothalamus and can regulate GnRH secretion (Boukhliq *et al.*, 1995).

2.3.1.1. THE EFFECT OF NUTRITION ON OVULATION RATE.

Ovulation rate is the one of the most important factors influencing reproductive performances in mammal. Oestrus activity can be suppressed and anoestrous can be induced by severe undernutrition, but ovulation rate is never completely suppressed (Rhind, 1992). Chronic undernutrition inhibits the tonic secretion of LH, possibly due to increased sensitivity to steroid negative feedback or other hypothalamic influences (Foster and Oster 1985, McShane and Keisler, 1991).

Food deprivation in hamster for 48h, starting just after the termination, prevents LH surge, ovulation and oestrus behaviour (Morin, 1986). Probably, food deprivation in murine, disrupts secretion of oestradiol and progesterone necessary to induce oestrus behaviour (Wade *et al.*, 1996). The incidence of oestrus is affected by the body fat reserves of an animal. Fasting induced anoestrous occurs more rapidly in lean hamsters than in fat hamsters (Schneider and Wade, 1989). Rats maintained on a high fat diet come into first oestrous earlier than do rats on a low fat diet. The factor which conveys body fat level to the reproductive system is Leptin, a 167- amino acid protein hormone secreted from adipocytes (Cunningham *et al.*, 1999). Leptin circulates in the plasma in proportion to body adiposity. Low leptin levels acts as a metabolic gate to inhibit the activity of the neuroendocrine reproductive axis in the male and female (Cunningham *et al.*, 1999). Circulating leptin levels decrease with fasting in rodents and primates resulting in decreased gonadotrophin secretion. Administration of leptin to these animal increased secretion of LH and FSH (Cunningham *et al.*, 1999). Yu *et al.*, (1992) reported that leptin stimulated gonadotrophin release from rat pituitaries *in vitro* indicating a direct effect of the leptin on pituitary. Leptin has also been shown to stimulate GnRH secretion from the

basal hypothalamus but, it is not certain that GnRH neurones express the leptin receptor (Cunningham *et al.*, 1999). Probably, leptin indirectly affects GnRH secretion *via* an effect on proopiomelanocortin and neuropeptide Y neurones in the arcuate nucleus. These neurones have leptin receptors and they effect the function of GnRH neurones.

In female mammals, reproduction is extremely sensitive to the availability of oxidizable metabolic fuels. Rapid suppression of glucose and fatty acids oxidation by 2-deoxy-D-glucose and methyl palmoxirate suppresses pulsatile release of LH in rat and mice (Wade *et al.*, 1996). The neural mechanisms controlling pulsatile LH release can respond to rapid changes in energy availability.

Glucose and fatty acid oxidation have been reported to be increased by leptin which results in an increase in gonadotrophin secretion. Therefore, leptin promotes fertility at the neuroendocrine level by increasing gonadotrophin secretion (Schneider *et al.*, 1998; Zachow *et al.*, 1999). On the other hand, leptin antagonizes the stimulatory effects of TGF- β on FSH dependent oestradiol production from rat, human and bovine granulosa cell by abolishing TGF- β effect on P450arom mRNA expression (Zachow *et al.*, 1999).

In sheep, the ovulatory response to nutrition depends on genotype; prolific breeds, such as the Finnish Landrace, have high ovulation rates irrespective of nutritional state, but breeds, which are not prolific and maintained under conditions of minimal management in a climatically harsh environment, show good ovulatory responses to nutrition (Rhind, 1992).

Nutritional effects on ovulation rate, in sheep, have been classified into two categories known as the static and dynamic effects. The static effect is a long-term effect of body condition and body weight on ovulation rate (Landau *et al.*, 1995), they influence ovulation rate by effecting the number of large follicles available for selection (Li *et al.*, 1995). The dynamic effect is a short-term effect of energy and protein supply on ovulation rate. Feeding high energy and protein supplements (such as Lupins), prior to mating, increases ovulation rate. It has been reported that, lupin supplementation, before the mating, increases ovulation rate, in Merino ewes, independently of changes in live weight (Parr *et al.*, 1992). Lupin supplementation before mating increased the

plasma concentration of insulin and prolactin but the concentration of LH and FSH did not change (Downing *et al.*, 1995a). Increased insulin and prolactin concentrations suggest that an increased supply of energy, to the follicle, mediates nutritionally stimulated increases in ovulation rate (Teleni *et al.*, 1984; Downing *et al.*, 1995). In ewes given supplementary lupins, plasma concentrations of glucose, acetate and propionate increased (Teleni *et al.*, 1989; Pearse *et al.*, 1994). Glucose oxidation, entry rates of glucose, acetate and CO₂ production also increased (Teleni *et al.*, 1989). It has been confirmed that, short-term administration of the energy-yielding substrates, glucose or acetate, can produce ovulatory responses of a similar magnitude to those elicited by feeding supplements of lupins (Teleni *et al.*, 1989).

2.3.1.2. THE EFFECT OF PRE-MATING NUTRITION ON OVULATION RATE

High nutrition prior to mating increases ovulation rate in sheep (Wilkins, 1997) and in pig (Ashworth *et al.*, 1999). Pig, that consumed a high pre-mating diet, had more corpora luteal than those fed at maintenance (Ashworth *et al.*, 1999). The reason, for increased ovulation rate, because of pre-mating nutrition, is not well understood. There are evidences that pre-mating nutrition may have an indirect effect on the ovary due to the changes in gonadotrophin secretion or it may have a direct effect on the ovary. Increased ovulation rate also can be a result of the alterations in endocrine changes. Pre-mating nutrition, in pig, has been confined with the increases in gonadotrophin secretion (Flowers *et al.*, 1989). Pre-mating nutrition has been reported to alter peripheral progesterone concentration. Ewes fed the low nutrition diet had higher progesterone level (Lazano *et al.*, 1998). In sheep, changes in peripheral progesterone concentration was primarily attributed to differences in the hepatic clearance rate of progesterone (Parr, 1991). In pig, no relation was found between the pre-mating nutrition and progesterone secretion as well as hepatic function in terms of liver weight or cytochrome P₄₅₀ activity (Ashworth *et al.*, 1999).

Recently, the effects of dietary levels of micronutrients, particularly vitamins, on reproductive performance have been studied. In pig, vitamin A, prior to mating, have been reported to increase progesterone secretion (Whaley *et al.*, 1997).

2.3.1.2.1. THE EFFECT OF NUTRITIONALLY INDUCED LIVE WEIGHT DIFFERENCES ON OVULATION RATE.

Ovulation rate is much more closely related to liveweight per se than to differences in liveweight. It has been suggested that, the ovarian response to the body reserve status of the ewe is greater than the response to the current plane of nutrition (Morley *et al.*, 1978). As mentioned before, improving feeding levels in the short term, before mating, is unlikely to improve lambing percentages, unless the ewes are initially in poor body condition (Morley *et al.*, 1978).

Variation in ovulation rate induced by liveweight is mediated through changes in preovulatory follicles during the follicular phase. Nutritionally-induced changes in liveweight have been reported to influence ovarian follicular volume and the total content of oestradiol and inhibin (Boukhiq *et al.*, 1996). It was reported that the ovaries of heavy ewes had more oestrogenic, potentially ovulatory follicles than ovaries of lighter ewes (Boukhiq *et al.*, 1996). These changes in the patterns of follicular development, due to nutritionally induced differences in liveweight, are reflected as variations in ovulation rate.

Changes in follicular development were reported to be independent of circulating levels of FSH (Xu *et al.*, 1989) but, Boukhiq *et al.*, (1996), reported that heavy ewes (ovariectomised and treated with oestradiol and inhibin) were able to maintain similar concentrations of FSH as light ewes for 4 days after ovariectomy but, then the increase in FSH concentration was more rapid in the heavy ewes. They proposed that FSH increased, after 4 days, because of escape from the suppression of ovarian steroids. This suppression was greater in light ewes because these of higher concentration of oestradiol receptors in the pituitary gland. Therefore, they concluded that nutrition altered the balance between pituitary FSH secretion and gonadal feedback by enhancing responsiveness to the inhibitory feedback effects of oestradiol and inhibin (Boukhiq *et al.*, 1996).

2. 3.1.3. THE EFFECT OF NUTRITION ON FERTILITY

Fertility is effected by food availability. It is suppressed when nutrition is poor and it is restored when adequate nutrition becomes available. Experiments, on hamsters, showed that food deprivation disrupted oestrous behavior due to the disruption of oestradiol and progesterone secretion necessary to induce oestrous

behavior (Dickerman *et al.*, 1993; Powers *et al.*, 1994; Wade *et al.*, 1996). Wade *et al.*, (1996) postulated that metabolic fuel manipulation by food deprivation, suppressing the oxidation of glucose and fatty acids by administration of 2-deoxy-D-glucose and methyl palmoxirate also partly affect neural oestrogen receptor immunoreactive cells in the ventromedial nucleus of the hypothalamus resulting with the inhibition of sexual receptivity in rodents.

Food availability affects all levels of the hypothalamic-pituitary-ovarian axis but the primary locus of altered function in nutritional infertility is thought to reside at the level of GnRH secreting neurones (Wade *et al.*, 1996).

Under-nutrition apparently inhibits pulsatile secretion of LH by reducing GnRH secretion by the hypothalamus. Suppression of LH pulse frequency impairs reproductive activity by reducing the growth rate of ovarian follicles to the preovulatory stage. There is a possibility that, pulsatile GnRH release is regulated by specific metabolic hormones that reflect nutritional status (Schillo, 1992). Experiments showed that during the first few hours after missing a meal, the circulating concentrations of insulin and thyroid hormone decreased, whereas the concentrations of cortisol and growth hormone increased in male rhesus monkeys (Dobey *et al.*, 1986; Cameron and Nosbisch, 1991). Feeding, after a brief period of fasting leads to rapid rises in circulating glucose and insulin concentrations, increases in tri-iodothyronine and decreases in growth hormone and cortisol (Williams *et al.*, 1996; Cameron, 1996). Glucose is used as an energy source within the body and neural mechanisms controlling gonadotrophin secretion respond to minute to minute changes in energy availability rather than body fat content or fat to lean ratio (Wade *et al.*, 1996). Therefore, glucose exerts an acute regulation on fertility whereas leptin appears to causes a long term regulation.

The pulsatile secretion of GnRH is readily suppressed by food restriction, but it returns rapidly when food restriction is alleviated. Experiments on rhesus monkeys suggest that the diurnal pattern of LH and testosterone secretion is regulated by food intake because the rise in LH and testosterone occurs just after feeding and is not associated with the timing of lights on and off (Cameron, 1996; Bronson, 1998).

Fertility is also affected by the failure of fertilisation, embryo mortality, lactational and seasonal anoestrus, impaired libido and spermatogenesis. Fertilisation, like ovulation, has a negligible nutrient requirement and therefore is likely to be

influenced only by extremes of nutrition. Specific dietary components can however effect fertilisation and the ingestion of oestrogenic pastures such as red clover or subterranean clover can impair fertility (Kallela *et al.*, 1984).

Severe undernutrition in early pregnancy increases the rate of early embryo mortality. Ewes fed below maintenance from mating to day 35 of pregnancy produced embryos that were reduced in weight (Parr and Williams, 1982) and ewes fed at 0.25 maintenance, for 21 days after mating, had embryos that were retarded in their development. In both cases embryo survival was not decreased, and subsequent experimentation indicated that these effects could be readily reversed. Birth weights were not reduced in ewes underfed for the first 35 days of pregnancy (Parr and Williams, 1982).

There have been consistent reports of increased embryo wastage in well fed or in overweight ewes (Parr *et al.*, 1987). This phenomenon appears to be related to an increased rate of metabolism of progesterone by the liver of well-fed ewes. This reduces the circulating levels of progesterone and increases embryo mortality. The sheep embryo is particularly sensitive to reductions in the concentrations of peripheral progesterone over a 48h period on days 11 and 12 of pregnancy (Parr, 1992). The pregnancy rate in ewes fed a high ration from days 2-14 after mating was significantly reduced compared with that of ewes fed a medium or low ration during the same period. In ewes fed a high ration, exogenous progesterone, given via an intravaginal controlled drug release device between day 8 to 14 after mating, increased pregnancy rate from 48 to 76% (Parr, 1992).

Progesterone treatment did not influence pregnancy rates in ewes fed medium or low rations (Parr, 1992). Therefore, it was suggested that, the level of feeding in early pregnancy should be maintained at or about maintenance level.

2. 3. 1.3.1. THE EFFECT OF GLUCOSE ON FERTILITY

The intravenous infusion of glucose into sheep has been reported to increase ovulation rate to the same degree as feeding lupin grain (Teleni *et al.*, 1989). Merino ewes infused intravenously 9 days before ovulation, with glucose or glucose and acetate together had ovulation rates higher than a group fed high protein in the form of casein. There was no difference in ovulation rate between sheep fed lupin grain and infused with glucose (Teleni *et al.*, 1989).

Glucose as an energy source is not essential for early-stage embryos and the timing of glucose utilisation differs from species to species (Rieger, 1991). In pigs, sheep and cattle, the use of glucose via glycolysis or the pentose phosphate pathway is relatively low (Bruck and Hyland, 1991; Wales and Waugh, 1993) in embryos at early cleavage stages but it increases during embryonic development (Azuma *et al.*, 1995). Both the embryo and its membranes actively utilise glucose by both glycolytic and oxidative pathways (Wales and Waugh, 1993).

As the development of sheep conceptus progresses, there is an increasing dependency on glycolytic pathways for ATP generation from glucose (Du and Wales, 1993).

2.3.1.3.2. THE EFFECT OF AMINO ACIDS ON FERTILITY.

Dietary amino acids have the potential to modulate reproductive function by stimulating a variety of metabolic changes which provide a signal to reproductive axis. Consumption of high protein diets elevate brain concentrations of branched-chain amino acids, histidine, methionine, tyrosine as well as neurotransmitters dopamine and noradrenaline (I'Anson *et al.*, 1991). Methionine supplementation, in dairy cows, have been reported to increase conception rate (Spiekers and Pfeffer, 1990).

Excitatory amino acid (EAA) neurotransmission plays an important role in reproduction. Glutamic acid is the major representative of the EAA system and it stimulates GnRH release from the arcuate nucleus of median eminence fragments *in vitro* via N-methyl-D-aspartic acid (NMDA) and/or d,l- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype receptors in the hypothalamus (Donoso *et al.*, 1992; Kalra *et al.*, 1997). Gamma-aminobutyric acid (GABA) is produced by the hypothalamus and is released episodically by the medial preoptic area. There is some evidence that it stimulates GnRH secretion from median eminence and the arcuate nucleus of male and female rats (Kalra *et al.*, 1997). Gamma-aminobutyric acid and glutamic acid have additive effects in inducing release of GnRH from arcuate nucleus or median eminence fragments *in vitro* (Donoso *et al.*, 1992).

Aspartic acid is endogenously present in the hypothalamus and brain which is a neurotransmitter in central nervous system (CNS) acting via the glutamate receptor and the analogue N-methyl-D,L aspartic acid (NMA) to stimulate GnRH secretion

leading to increased gonadotrophin secretion in rodents, primates and sheep (Carbone *et al.*, 1992; Downing *et al.*, 1996). Intra-peritoneal injection of the rat with aspartic acid induced LH release *in vivo* (D'Aniello *et al.*, 2000). Under *in vitro* conditions, aspartic acid was able to induce LH release from the pituitary only when this gland was co-incubated with hypothalamus. This is because aspartic acid increased LH release through hypothalamic GnRH release (D'Aniello *et al.*, 2000).

N-methyl-D-aspartate is present in the hypothalamus. N-methyl-D-aspartate is the methylated form of aspartic acid and biochemically is very similar to aspartic acid (D'Aniello *et al.*, 2000). In *in vitro* treatment with N-Methyl-D-aspartate (NMDA) significantly increased GnRH release from anterior preoptic area and medial basal hypothalamic area of the rat (Arias *et al.*, 1996). Systemic administration of NMDA stimulates secretion of LH in adult female rats (I'Anson *et al.*, 1991; Arias *et al.*, 1996). Therefore, excitatory amino acid neurotransmission is important factor influencing hypothalamo-pituitary- ovarian axis and modulating secretion of gonadotrophins. Gonadotrophins are an essential factors in diet-induced infertility since gonadotrophin administration prevented infertility in fasted mice (I' Anson *et al.*, 1991). Treatment of dairy cows with GnRH increased pregnancy rate at the time of insemination (von Straaten *et al.*, 1997).

In ruminants, the influx of amino acids into peripheral circulation may contribute to insulin release and insulin-mediated glucose uptake resulting with an increase in ovulation rate (Downing *et al.*, 1995). In sheep, rapid suppression of glucose and amino acid utilisation, by insulin or 2-deoxy-D-glucose treatment, immediately suppresses pulsatile LH release (Wade *et al.*, 1996). In mature mice, hypoglycaemia because of insulin treatment has been reported to be a primary factor inducing infertility (McClure, 1967).

It was reported that, insulin-induced hypoglycaemia decreases oestradiol-induced gonadotrophin secretion in anoestrous ewes (I' Anson *et al.*, 1991). Gonadal steroids modulates signaling processes within hypothalamus which facilitate cross-talk among various components of neural system effecting secretion of GnRH. The neurons elaborating regulatory messenger molecules are the targets of gonadal steroids. Therefore, ovarian steroids promote the synthesis, storage and release of these regulatory messenger molecules including both excitatory (NeuropeptideY, galanin,

excitatory amino acid and neurotensin) and inhibitory types (β -endorphin, neuropeptide K and GABA) (Kalra *et al.*, 1997).

2.3.1.4. EFFECT OF NUTRITION ON METABOLIC FUNCTION

Nutritionally induced changes in plasma substrate concentrations can affect the secretion of metabolic hormones. This may alter substrate uptake and tissue metabolism including those of the reproductive axis. Changes in metabolic status can mediate short-term nutritional effect on reproductive function.

Experiments on prepubertal gilts showed that feeding to appetite, following feed restriction, increased follicular fluid volume, uterine weight and ovarian weight (Booth *et al.*, 1994). Feed restriction can increase the releasable pool of pituitary LH by inhibiting release, but not the synthesis of LH in prepubertal gilts (Armstrong and Britt, 1987) and sheep (Booth *et al.*, 1994). Feed restriction in the prepubertal ewe and heifer and in the female rat (Lively and Piacsek, 1988) suppressed episodic LH secretion. This suppression appears to be mediated partly by a fall in pituitary responsiveness to GnRH. Administration of GnRH to dietary restricted male rhesus monkeys will restore normal LH secretion (Dubey *et al.*, 1986; Cosgrove *et al.*, 1995). In feed restricted lambs, administration of EAA agonist, N-methyl-D-aspartate (NMDA), increased episodic secretion of LH (Ebling *et al.*, 1990).

The neuro-endocrine centres governing the release of GnRH seem to be especially sensitive to the availability of energy substrates, because reduction of available oxidizable metabolic substrates decreased pulsatile LH release (Miller *et al.*, 1998).

In male sheep, nutritional supplementation has been shown to increase peripheral and cerebro-spinal fluid (CSF) concentrations of glucose and insulin as well as gonadotrophin secretion (Boukhliq *et al.*, 1996b), but short term administration of glucose and insulin did not increase gonadotrophin secretion (Boukhliq *et al.*, 1996b). The reason for this might be a reduced level of glucose transporter at the blood brain barrier.

It has been also suggested that, nutritionally induced increases in ovulation rate, can result from changes in oestradiol metabolism in the liver, causing a reduction

in feedback inhibition on the hypothalamus which in turn results in increased release of FSH (Payne *et al.*, 1991).

2.3.1.4.1. NUTRITIONAL EFFECTS ON HEPATIC FUNCTION AND METABOLISM.

Feeding levels alter the transport of digested nutrients from the gut to the rest of the body and therefore alters the intake of metabolizable energy. The hepatic enzymes, cytochrome b₅, and NADPH-cytochrome -c reductase concentrations have been found to be slightly higher in ewes with a high nutritional state (Smith *et al.*, 1990).

Short-term nutrient restriction was reported to alter the metabolic activity of liver primarily affecting its size (Ferrell and Koong, 1985). Liver accounts for approximately 1.5-2.5% of body weight. Liver weight of wether lambs fed *ad-libitum* for 21 days increased whereas liver weight of lambs fed at maintenance did not (Burrin *et al.*, 1990). The changes in liver weight were greater than for other visceral organs (stomach and small intestine). During the 21 days, O₂ consumption by the liver was increased by *ad libitum* feeding (Burrin *et al.*, 1990). This increase in O₂ consumption is the result of increased liver size rather than increased tissue specific metabolic activity (Burrin *et al.*, 1990).

A positive linear relation has been proposed between metabolizable energy intake, hepatic blood flow and O₂ conception in cows and sheep (Kelly *et al.*, 1993). Substrate cycling within the liver accounts for 18 to 30.3% of total O₂ consumption. During starvation substrate, cycling decreases (Blum and Rabkin, 1986).

The liver accounts for approximately 20% of whole body O₂ consumption. Urea synthesis by the liver depends on the type of diet, the level of intake and pathological state. Urea synthesis requires considerable amount of energy approximately 4 moles of ATP/ mole urea and oxygen (McBride and Kelly, 1990).

Hepatic parenchyma cells have the unique ability of catabolizing cholesterol into bile acids. This is the initial process through which cholesterol is eliminated from the body. Bile acid synthesis is derived from a metabolically active pool of free cholesterol from the newly absorbed lipids or from plasma lipoproteins. The conversion of oestradiol to bile acid is limited by 7 α -hydroxylase activity which is a cytochrome P₄₅₀ dependent mono-oxygenase in the liver. The activity of this enzyme is

directly inhibited by oestradiol and progesterone. Progesterone is the more potent inhibitor of this activity than oestradiol (Chico *et al.*, 1994). Increased oestradiol metabolism in the liver results in a decrease in the plasma levels of oestradiol and reduced feed back effect on the hypothalamus, altering the secretion of FSH (Payne *et al.*, 1990; Chico *et al.*, 1994).

The liver is the primary source of blood glucose and it plays a central role in maintaining blood glucose levels. An increase in feeding level causes an increase in glucose concentration in blood through a decrease in Glucose-6-Phosphatase activity, whereas fasting increases the activity of Glucose-6-Phosphatase. Increased activity of this enzyme decreases hepatic glucose production due to decreased availability of substrate (Barzilai *et al.*, 1995). Increased glucose levels in blood increase ovulation rate by supplying energy substrates to follicles. Therefore, manipulating the activity of hepatic microsomal enzymes may alter ovulation rate in sheep (Smith *et al.*, 1990; Payne *et al.*, 1991).

The liver produces 82% of blood glucose. Intravenous infusion of sheep with glucose increased blood glucose and insulin levels, but decreased glycogenolysis in the liver. This decrease in hepatic glucose production is a direct effect of insulin on hepatic glucose production (Freetly and Klindt, 1996). Hepatic oxygen consumption was also decreased by glucose infusion (Freetly and Klindt, 1996).

When male Wistar rats were fed an energy dense diet, the hepatic mitochondrial NADH/NAD⁺ ratio increased (Iossa *et al.*, 1995). This increase in NADH/NAD⁺ ratio caused a significant decrease in the transport of reducing equivalents into the mitochondria. The increase, in NADH/NAD⁺ ratio, was attributed to decreased NADH oxidation by the respiratory chain. This decrease in NADH oxidation is probably due to a decreased NADH dehydrogenase activity rather than to an impairment of ATP synthesis. The oxidative capacity of hepatic mitochondrial NADH is also responsive to hormonal factors such as norepinephrine, whose hepatic turnover increases during overfeeding. Hepatic metabolic efficiency is decreased by an energy dense diet because a greater fraction of cytoplasmic NADH transported to mitochondria through the α -glycophosphate shuttle as FADH₂ (Iossa *et al.*, 1995). Since FADH₂ enters the electron transport chain, after the first reaction, which translocates a hydrogen ion, FADH₂ oxidation produces less of a gradient for driving

ATP synthesis. The result is a decrease in hepatic metabolic efficiency, in rats fed with an energy dense diet (Iossa *et al.*, 1995).

2.3.1.4.2. NUTRITIONAL EFFECT ON BASAL METABOLISM.

Biochemical processes such as Na^+ , K^+ -ATPase activity, protein synthesis and degradation, substrate cycling and urea synthesis in the gastro-intestinal tract and liver accounts for about 23% of whole body O_2 consumption and ATP utilisation. These biochemical processes are influenced by dietary composition, level of intake, age and metabolic state.

The level of nutrition will alter visceral organ growth, blood flow and metabolic activity. Increases in organ mass are associated with higher levels of organ O_2 consumption. Heat production and whole-body O_2 consumption increase when the level of feeding increases (Ortigue and Durand, 1995). Experiments on non-lactating pregnant ewes, subjected 3 different feeding regimes, fasted, maintenance and twice maintenance showed that increasing levels of intake caused an elevation in whole-body O_2 consumption and hepatic blood flow (Kelly *et al.*, 1993). Hepatic portal blood flow is directly proportional to level of intake and was lowest in fasted ewes, intermediate for maintenance and highest for twice maintenance levels of feeding.

The consumption of O_2 by the portal-drained viscera (PDV), was reported to vary directly with the level of food intake in steers and lambs (Burrin *et al.*, 1989; Reynolds *et al.*, 1991). Increased PDV O_2 consumption after feeding may be the result of increased Na^+ , K^+ -ATPase dependent respiration which is a major component of cellular energy expenditure (McBride and Kelly, 1990). In sheep, PDV O_2 consumption accounts for 22-28% of whole body O_2 consumption (Reynolds *et al.*, 1991; Kelly *et al.*, 1993). In fasting ewes, PDV O_2 consumption increased as a percentage of whole body O_2 consumption (Eisemann and Nienaber, 1990) because of a decrease in tissue specific metabolic activity relative to the decrease in PDV O_2 uptake (Kelly *et al.*, 1993; Ortigue and Durand, 1995).

2.4. THE MECHANISM OF NUTRITIONAL EFFECTS ON OVULATION RATE AND FERTILITY

2.4.1. NUTRITIONAL EFFECTS ON THE FOLLICLE

Various degrees of nutritional deprivation, in cattle, before or after calving will affect the patterns of ovarian follicle development. Moderate levels of underfeeding during this period are more likely to affect the mechanisms of final follicle maturation and ovulation, whereas greater deficiencies in nutrition appear to effect the mechanisms regulating the size and the dynamics of growth and maturation of the dominant follicle. Feeding cows, *ad libitum*, after a period of anovulation will increase the diameter and persistence of successive dominant follicles and the development of these follicles was proportional to body mass (Murphy *et al.*, 1990).

Nutritional effects on the follicle may be a consequence of altered gonadotrophin secretion or altered sensitivity of the follicle to LH and/or FSH stimulation (Jolly *et al.*, 1995).

2.4.2. NUTRITIONAL EFFECTS ON THE HYPOTHALAMO-PITUITARY AXIS

In 1930, experiments in male rats showed that pituitaries of starved animals had low gonadotrophic activity (Mason and Wolfe, 1930). This led in the 1940's, to the idea that acute or prolonged food restriction led to a marked decrease in the function of ovary and testis due to decreased secretion of gonadotrophins (Mulinos and Pomerantz, 1940).

Nutritional stimulation of gonadotrophin secretion is primarily accompanied by changes in the concentrations of glucose and insulin in blood and cerebrospinal fluid (CSF) (Miller *et al.*, 1995). Nutrition may also affect gonadotrophin secretion through dietary amino acids. A diet known to increase gonadotrophin secretion increased plasma and CSF concentrations of Serine and Threonine on day 10 of experiment (Miller *et al.*, 1998). This diet increased LH pulse frequency and FSH concentrations on day 11 of the feeding regime, but not LH pulse amplitude and mean plasma LH concentration (Miller *et al.*, 1998).

GnRH neurones may be directly sensitive to changes in metabolic status. The cell surface of GnRH neurones possess cilia and the neurones may possibly receive

different metabolic signals through these cilia (Hooper and Moulins, 1989). These signals may directly affect the GnRH pulse generator and the release of GnRH as a consequence of rapid changes in diet (Cameron and Nosbisch, 1991).

Food restriction in female rats has been shown to diminish the number and size of LH and FSH immunopositive cells and pituitary gonadotrophin content (Root and Russ, 1972). This finding was confirmed by the administration of GnRH to fasted animal (Kotsuji *et al.*, 1992). Administration of GnRH to these animals increased the size of both LH and FSH-positive cells. This led to another hypothesis; that fasting reduced the hypothalamic release of GnRH (Kotsuji *et al.*, 1992). Exogenous GnRH is required for the physiological maintenance of genomic expression of both α and FSH β -subunit mRNA (Weiss *et al.*, 1992). Therefore, the loss of gonadotrophin gene expression during starvation is the result of decreased hypothalamic release of GnRH (Bergendahl *et al.*, 1991). A similar result was seen in adult male rhesus monkeys. In these animal, when there is no dramatic loss in body weight, less than one day of starvation significantly inhibited pulsatile LH and testosterone secretion (Cameron and Nosbisch, 1991). The frequency of pulsatile LH secretion decreased significantly, but there was no change in LH pulse amplitude (Helmreich *et al.*, 1993). External administration of GnRH increased circulating levels of gonadotrophins in the starved animal (Dubey *et al.*, 1986).

Food deprivation in sheep decreased pulse frequency of LH pulses, although the amplitude of LH pulses did not change (Foster *et al.*, 1989). Serum concentrations of LH decreased from between 92% and 48%. During 4-6 days of fasting, the levels of α and β subunit mRNA decreased, possibly a result of decreased hypothalamic release of GnRH. Probably, GnRH neurones are primarily affected by food deprivation. Because administration of GnRH to fasted rat restored pulsatile LH release. It was reported, by Wade *et al.*, (1996), that neural mechanisms controlling pulsatile LH release can give a rapid response to energy availability. Inhibition of fatty acid and glucose oxidation, by chemicals, immediately suppresses pulsatile LH release.

Undernutrition of cycling goats has been reported to decrease the preovulatory surge of gonadotrophins resulting in a decreased incidence of ovulation (Mani *et al.*, 1996).

2.4.2.1. THE EFFECTS OF GLUCOSE AND ENERGY INTAKE ON LH AND FSH SECRETION

Energy and protein in food effect secretion of gonadotrophins. There are many studies concerning the relation, between the availability of energy (glucose and fatty acids) and the gonadotrophin secretion.

Supplementation of the diet of mature rams with lupin grain increased the concentration of glucose in the peripheral circulation and in the Cerebro Spinal Fluid (CSF). After 10 days of supplementation with lupin grain, there was an increase in the LH pulse frequency and the mean FSH concentration (Boukliq *et al.*, 1996b), while intravenous infusion of glucose did not effect the secretion of LH and FSH, but increased the blood levels of insulin and glucose (Boukliq *et al.*, 1996b). That is because short term infusion of glucose did not change the concentration glucose in CSF and this did not effect the function of GnRH secreting neurones. Increased insulin concentration, during the infusion of glucose, may induced hypoglycaemia resulting with a decrease in gonadotrophin secretion (Downing *et al.*, 1995c).

Glucose availability plays an important role in the secretion of LH. The hypothalamo-hypophyseal axis of female rats is more sensitive to decreased glucose availability (Nagatani, 1996). In male and female rat, inhibition of glycolysis, by 2-deoxyglucose, decreased secretion of LH (Nagatani, 1996; Wade *et al.*, 1996). In rat, injection of 2-deoxyglucose (2DG) to the 4th ventricle of the brain with higher doses (40mg/kg) suppresses pulsatile LH secretion (Murahashi *et al.*, 1996).

2.4.2.2. THE EFFECT OF DIETARY PROTEIN AND AMINO ACID INTAKE ON LH AND FSH SECRETION.

Feeding Coopworth ewes with a high protein diet, starting on day 7 of the oestrus cycle increased the concentration of plasma FSH on days 7, 13 and 18 (Smith *et al.*, 1990).

Lupin grain provides a source of highly digestible energy and protein. Mature rams, fed for 10 days, with lupin grain, had an increased LH pulse frequency and mean FSH concentration (Boukhliq *et al.*, 1996).

The post-ruminal infusion of sheep with energy and protein (equal to the energy and protein content of lupin grain) increased LH pulse frequency within 5-10 days, but this increase in LH pulse frequency was smaller than when sheep received

lupin grain (Blache *et al.*, 1996). There was no differences in FSH. Therefore it was concluded that, effect of diet on the secretion of gonadotrophins is not entirely dependent on the total energy and protein content of the diet, but possibly also on differences between specific components of the proteins, carbohydrates and fats within the diet (Boukhliq *et al.*, 1996; Blache *et al.*, 1996).

It has been hypothesised that, the relationship between nutritional state and ovulation rate may involve the action of specific nutrients such as amino acids, on gonadotrophin release (Downing *et al.*, 1995b)

The excitatory amino acids are neuroendocrine regulators of pituitary gonadotrophin secretion. These amino acids are found in large concentration in many hypothalamic nucleus (figure 2.4.2.2.1).

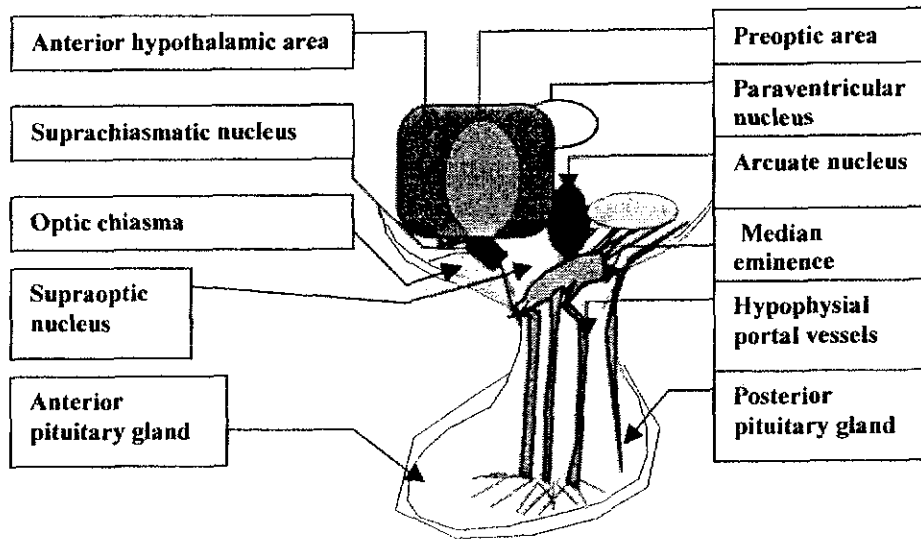


Figure 2.4.2.2.1.; Excitatory amino acid neurotransmission is an important factor in the release of GnRH and the regulation of LH and FSH secretion (Brann and Mahesh, 1991; Lopez *et al.*, 1992). Excitatory amino acids are found in the suprachiasmatic nucleus, preoptic area, paraventricular nucleus, arcuate nucleus and supraoptic nucleus (Brann and Mahesh, 1994). The neurones of these hypothalamic nuclei transfer their neurohormones through their axon projecting to the median eminence where they cause the release of GnRH. Not all of the neurones of these hypothalamic centres act via pre-optic area, the arcuate nucleus and median eminence. Some of them (neurones of paraventricular nucleus) act via the supraoptic nucleus and the posterior pituitary gland to initiate hormone secretion (Brann and Mahesh, 1994).

2.5. FOLLICLE CULTURE

2.5.1. NUTRITION OF THE FOLLICLE

The survival and viability of cultured follicles is influenced by nutrients available in the culture media. *In vitro* maturation of oocytes within the follicle influenced by the availability of energy, amino acids and oxygen.

2.5. 1. 1. ENERGY INTAKE AND ATP PRODUCTION.

Under *in-vitro* conditions, the meiotic response of oocytes depends upon the culture media and the energy substrates contained within. In cultured follicles, ascorbic acid uptake and transport depends on the energy and concentrations of some ions (e.g.; Na⁺). These energy-dependent transporters are positively regulated by FSH, LH and GnRH in the follicle (Behrman *et al.*, 1996).

Lactate is major product of follicular carbohydrate metabolism. In culture, mouse follicles produce large amount of lactate as a result of increased metabolic activity of FSH stimulated granulosa cells. A high rate of lactate production by anaerobic glycolysis is a phenomenon exhibited by a normal cells with proliferative potential. This is thought to be the fastest route for supplying the large amounts of ATP required for rapid growth and steroidogenesis (Boland *et al.*, 1993).

Mouse oocytes cultured with somatic cells, undergo maturation *in-vitro* in the presence of glucose and lactate as the sole energy source (Biggers *et al.*, 1967), but glucose and lactate do not support maturation of isolated oocytes (Biggers *et al.*, 1967). It was thought that the effect of altering glucose and pyruvate concentrations on oocyte maturation might be due to the differential activities of energy metabolising pathways and therefore differences in the production of ATP by oocyte-cumulus cell complexes. The ATP content did not change over 3h incubation, but it increased when the culture period was increased to 17-18h (Downs and Mastropolo, 1994). This increase in ATP production was independent of the presence of pyruvate (Downs and Mastropolo, 1994).

2.5. 1. 2. OXYGEN SUPPLY TO THE FOLLICLE

Oxygen enters to the follicle by diffusion and follicles consume oxygen in proportion to their radius. Most of the oxygen entering the follicle is consumed in the outer 5% of the radius, which corresponds to the outer layer of cells. Very little oxygen reaches the oocyte (Gosden and Byatt-Smith, 1986). Therefore, oxygen is primarily consumed by the granulosa cell (Gosden and Byatt-Smith, 1986; Boland *et al.*, 1993). Granulosa cells consume 0.7% of total O₂ consumed by ovary (Baird *et al.*, 1973). Increasing the number of granulosa cell layers during follicle growth *in-vitro* requires an adequate level of oxygen for delivery to oocyte (Smitz *et al.*, 1996). Oxygen diffusion into the follicle undergoing antrum formation is probably greater than diffusion into preantral follicles because of attenuation of membrane granulosa and partly because of a presumed lack of oxygen in follicular fluid. Follicular fluid is evidently, not anoxic and the oxygen partial pressure is close to that of normal ovarian venous blood (Boland *et al.*, 1993).

2. 5. 1. 2. 1. OXYGEN SUPPLY TO FOLLICLES IN-VIVO

The granulosa cells surrounding the oocyte protect the oocyte from the harmful effect of high oxygen concentrations. It was reported that high oxygen levels effect maturation of the oocyte in a negative way and that this harmful effect is reduced in *in-vitro* conditions. Oxidative stress to the follicle may effect energy levels resulting in mitochondrial dysfunction and inhibition of the citric acid cycle. The response of follicle to oxidative stress is related to its capacity to supply reducing equivalents. The depletion of oxygen within the follicle results in accumulation of reducing equivalents due to the inhibition of oxidative phosphorylation (Lund *et al.*, 1994).

2. 5. 1. 2. 2. OXYGEN SUPPLY TO FOLLICLES IN- VITRO

Oxygen is an important factor for the maintenance of normal growth of the follicle *in vitro* and the level of oxygen in the culture medium plays an important role in follicle growth. Oxidative phosphorylation and cholesterol synthesis are oxygen-dependent processes going on within the follicle.

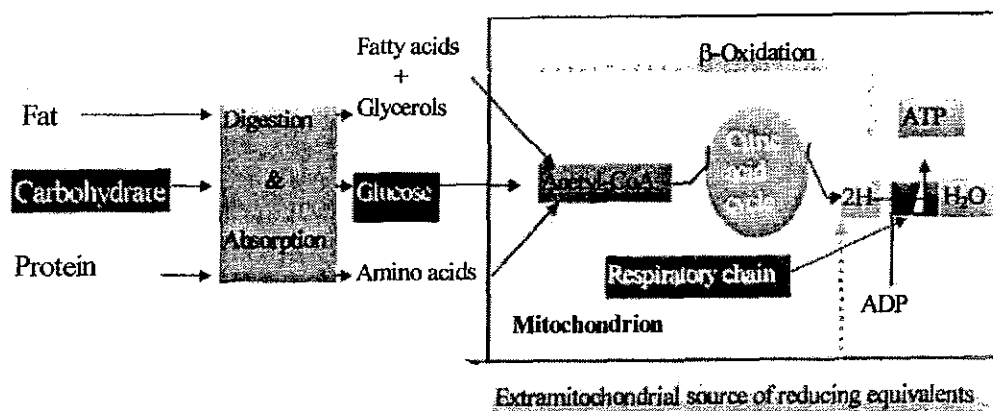
Simitz *et al.*, 1996 reported that a reduced oxygen level (5% O₂) inhibited normal follicular growth and differentiation and impaired oocyte maturation. On the other hand, nuclear maturation of the oocyte is adversely affected by high O₂

concentrations. Under culture conditions, oocytes are more likely to be affected by the detrimental effects of high concentration of O_2 because they are not well protected by the surrounding granulosa cells. Late in culture, this harmful effect of high O_2 concentration is reduced. Under normal oxygen levels (20%), mouse follicles maintained the germinal vesicle stage, whereas follicles, cultured in an oxygen reduced environment, underwent spontaneous germinal vesicle breakdown. High levels of O_2 , early in culture, reduced the percentage of oocytes undergoing germinal vesicle breakdown and cleavage to two cell stage, but late in culture, after germinal vesicle breakdown, this effect is partly reduced (Eppig and Wigglesworth, 1995).

Reduced oxygen damaged the morphological integrity of cultured follicles. There was a rapid active outgrowth of granulosa cells and an active outward migration of the oocyte-granulosa-cell complex through the basal lamina. Reduced oxygen levels decreased production oestradiol and inhibin. This reduction may originate from a reduced synthesis of cholesterol and a lowered mitogenic activity of granulosa cells.

2. 5. 1. 3. REDUCING EQUIVALENTS

All energy released by the oxidation of carbohydrate, fat and protein is made available in the mitochondria as reducing equivalents (hydrogen or electrons). The mitochondria contain a series of catalysts known as the respiratory chain. The respiratory chain consists of a number of redox carriers that proceed from a NAD-linked dehydrogenase system, through flavo-proteins and cytochromes to molecular oxygen. This respiratory chain represents a system for trapping liberated free energy as high-energy phosphate. Hydrogen and electrons are passed along the respiratory chain in sequence from electro-negative components to the electropositive to form water. High-energy phosphate is captured by ADP to produce ATP. The ATP uses this free energy to drive cellular processes that require energy (Figure 2. 5. 1. 3. 1.) (Mayes, 1996)



Figure; 2.5.1.3.1. Oxidation of major foodstuffs leads to the generation of reducing equivalent (2H) that are collected by the respiratory chain for oxidation and coupled generation of ATP (Adopted from Mayes, 1996).

Two coenzymes, Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP) known as the nicotinamide nucleotides, exist in oxidised (NAD^+ and NADP^+) or reduced (NADH and NADPH) forms (Lund *et al.*, 1994). They consist of pyrophosphate linked to a complex of adenine and nicotinamide nucleotides. Reduction of the coenzymes occurs on the nicotinamide ring, a dehydrogenase transfers two electrons and one proton from their substrates to the coenzymes and a second proton is liberated. This dehydrogenase reaction is pH dependent.

2.5.1.3.1. NICOTINAMIDE ADENINE DINUCLEOTIDE

Nicotinamide Adenine Dinucleotide is an electron carrier and it has a role in oxidative processes such as glycolysis, the citric acid cycle and β -oxidation of fatty acids (Figure 2.4.1.3.1.). It is mainly present in its oxidised form (Apps *et al.*, 1992; Lund *et al.*, 1994).

The conversion of pregnolone to progesterone takes place in the agranular endoplasmic reticulum (microsomes) by a microsomal enzyme complex, Δ^5 -3 β -hydroxysteroid dehydrogenase and Δ^{5-4} isomerase (Gore-Langton and Armstrong, 1994). This enzyme complex functions within the ovary and utilises NAD^+ as an electron acceptor

2.5.1.3.2. NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

Nicotinamide Adenine Dinucleotide Phosphate is also an electron carrier but, its metabolic functions different from NAD. It is predominantly present in the reduced form (NADP⁺). It has a role in the synthesis of fatty acids and sterols.

The enzyme systems that catalyses conversion of cholesterol to reproductive steroids, are located on the matrix side of the inner mitochondrial membrane. The enzyme systems are complexes comprising of three components: Cytochrome P-450_{acc}, which is a terminal electron acceptor; a Flavine Adenine Dinucleotide (FAD), containing a flavoprotein, and a sulphur-containing heme protein (luteodoxin), which serves to shuttle electrons between P450_{acc} and FAD (Gore-Langton and Armstrong, 1994). This reaction utilises NADP generated within the mitochondria by oxidation of Krebs cycle intermediates or fatty acids. Three moles of NADP and oxygen are utilised per mole of cholesterol undergoing side-chain cleavage. The conversion of cholesterol to pregnenolone takes place in the mitochondria, pregnenolone is the key steroidogenic intermediate common to all steroids produced by the follicle. The reaction, by which androstenedione and testosterone are converted to the oestrogens (oestrone and oestradiol-17 β) requires NADPH as the hydrogen donor (Apps *et al.*, 1992; Lund *et al.*, 1994). Conversion of progesterone(Δ^5) and pregnenolone (Δ^4) to androgen is catalysed by an enzyme complex, 17 α -hydroxylase: C-17,20-lyase, which are component of agranular endoplasmic reticulum requires NADP and molecular oxygen.

2.5.1.4. CHOLESTEROL UTILIZATION

Follicular steroids are produced from cholesterol. Cholesterol is derived from three possible sources.

- 1) Preformed cholesterol stored within ovarian cells, either as free cholesterol, a constituent of cell membrane or as cholesterol esters stored within cytoplasmic lipid droplets.
- 2) Preformed cholesterol taken up from blood, in the form of circulating lipoproteins.
- 3) Cholesterol synthesised *de novo* from acetate in the ovarian cell. Acetate is derived from metabolism of carbohydrate, fat or protein within the cell.

Extracellular lipoproteins bind via their apoprotein component of specific receptors on the cell membrane and are then internalised by endocytosis. Lysosomal enzymes degrade the lipoproteins into cholesterol and amino acids (Rajendran *et al.*, 1983; Gore-Langton and Armstrong, 1994).

Granulosa and luteal cells can obtain cholesterol for steroidogenesis through *de novo* synthesis and from circulating plasma lipoproteins (Savion *et al.*, 1982). It has been reported that, extracellular cholesterol rather than *de novo* synthesised cholesterol is the preferred substrate for ovarian steroidogenesis in most mammalian species (Schraiber *et al.*, 1982; Wang and Greenwald, 1984). Theca cells are more dependent than granulosa on the presence or absence of lipoproteins in the culture media (Gore-Langton and Armstrong, 1994). Thus, high and low density lipoproteins are the steroidogenic precursor for ovary and testis. Species differences occur in the relative importance of the two classes of lipoproteins. High density lipoproteins appear to be of greater quantitative importance in rodents, whereas cholesterol associated with low density lipoproteins are the major circulating form of steroidogenic cholesterol in other species including human (Gore-Langton and Armstrong, 1994).

2.5.1.5.AMINO ACID UTILIZATION.

Ovarian follicular cells readily take up amino acids and they also enhance amino acid uptake by the oocyte via gap junctions. Granulosa cells have an important role in amino acid uptake by oocyte from follicular fluid. The effect of follicular cells, on amino acid uptake by the oocyte depends on the stage of follicular development and the presence of other amino acids (Haghighat and Van Winkle, 1990; Jimena *et al.*, 1993). The uptake of L-Alanine, L-Lysine and choline into mouse oocytes, at the preantral stage, was enhanced by follicular cells. Leucine uptake into the oocyte was not enhanced when only this amino acid was present, but was enhanced when other amino acids were also in the medium (Haghighat and Van Winkle, 1990). Glycine uptake into the whole follicle at the preantral stage is predominantly Na⁺ dependent and its uptake was decreased by Serine, Leucine and Alanine (Haghighat and Van Winkle, 1990).

Addition of hormones into the culture media facilitates the transport of amino acids into the follicular cells because follicles, on their own, have an inhibitory effect on membrane transport of amino acids. It has been reported that the uptake of amino

acid across the plasma membrane can be induced by removing the follicular cells, but leaving cumulus cells intact or by gonadotrophin administration (Shalgi *et al.*, 1979; Crosby *et al.*, 1981). Therefore hormones, in culture, are important for the increase in amino acid uptake by follicular cells (Shalgi *et al.*, 1979).

Serine and Threonine amino acids are substituted by intra cellular proteins taking role in gene transcription, protein and steroid production. These amino acids are phosphorylated and this phosphorylation is important for the production of steroids *via* the expression of mitochondrial proteins (e.g. P450_{scc} enzyme) taking role in steroidogenesis (Stocco and Clark, 1993).

CHAPTER III: FOLLICLE CULTURE

3.1. THE GROWTH OF PREANTRAL FOLLICLES IN CULTURE MEDIA SUPPLEMENTED WITH SERINE/THREONINE

Abstract

This experiment was designed to test effect of Serine and Threonine, with or without Pregnant Mare Serum gonadotrophin (PMSG), on preantral follicle growth *in vitro*. Preantral follicles, 90-120 μm in diameter were dissected mechanically using sterile, fine needles from 21-day-old prepubertal mice. Follicles were cultured individually for seven days in media supplemented with α -MEM. Every twenty-four hours, follicle diameter was measured and the media changed. Amino acid and PMSG supplementation together did not cause any significant change in growth, but increased rate the of atresia after 48h. The addition of PMSG at 24h caused a further increase in follicle growth but this increase was not statistically significant. During the first 48h of culture, diameters of the follicles increased from 100 μm to 112.5 $\pm$ 2.2 (control), 115.7 $\pm$ 4.2 (Serine/Threonine), 122 $\pm$ 5.4 (PMSG) and 115 $\pm$ 4.4 (Serine/Threonine plus PMSG) respectively. The growth of follicles in this culture system was normal for the first day but, after the first day follicle, growth was reduced and the follicles became atretic.

INTRODUCTION

The *in vitro* production of gametes is important for the preservation of economically valuable genotypes. These techniques can help decrease the generation interval, increase the production of gametes and facilitate manipulation of the genome within the population.

Mouse follicles grow faster *in vitro* than *in vivo* (Hartshorne *et al.*, 1994). This is probably because the ovarian stroma exerts an inhibitory control over the initiation of follicle growth *in vivo* (Wandji *et al.*, 1996). Follicles cultured in-groups but without direct contact with each other grow more slowly than when cultured individually

(Nayudu and Osborn, 1992). Inhibition of growth, in cultured follicles, having contact is not uniform, some follicles grow faster while the others regress. This is possibly due to mechanisms of intra-ovarian follicle selection (Nayudu and Osborn, 1992).

Early follicular growth is independent of gonadotrophins (Eppig and O'Brien, 1996), but a basal level is required for normal development. Injection of hyposectomised mice with 250ng of follicle stimulating hormone (FSH) delayed atresia and maintained more large healthy antral follicles.

Pregnant Mare Serum Gonadotrophin (PMSG) is generally used as a source of gonadotrophins (FSH and LH). Administration of PMSG to adult rat increased mitosis of granulosa cells (Peluso and Steger, 1978) and reduced the rate of atresia in preovulatory large follicles. Injection of rat with PMSG, on oestrous increased the diameters of large healthy follicles and reduced the number of atretic large follicles (Braw and Tsafiriri, 1980). Atresia in rodents appears to play a central role for the selection of current number of follicles for ovulation. Treatment of rodent with PMSG increased expression of TGF- β expression in follicular cells and co-treatment with LH further increased expression of TGF- β (Ghiglieri *et al.*, 1995). Therefore, gonadotrophin supplementation in culture will meet the maintenance of the follicles and facilitate their growth due to the *de novo* production of TGF- β . Therefore PMSG treated follicles were kept as a positive control.

The effect of nutrition on follicle growth is not just a matter of the amount of energy and protein supplied to the animal but, also of other micro nutrients, such as amino acids. Under *in vivo* conditions, some amino acids, such as Arginine, are capable of altering the endocrine status of the animal (Recabarren *et al.*, 1996). Ovarian follicular cells readily take up amino acids and granulosa cells have an important role in amino acid uptake from follicular fluid (Jimena *et al.*, 1993).

Transforming Growth Factor Beta (TGF β) is produced in the ovary. Endogenous TGF β is localised in theca, granulosa and luteal cells (Mulheron *et al.*, 1991; Magoffin *et al.*, 1995). Receptors for this growth factor are present in granulosa cells of primordial and preantral follicles (Dorrington *et al.*, 1993). Under normal physiological conditions, TGF β is produced at optimum levels sufficient to maintain normal follicular function. The mouse oocyte expresses TGF β and it has an effect on early follicular growth (Dong *et al.*, 1996). Under *in vivo* condition, high doses (50-

500ng/ml) of TGF β reduced ovulation rate in mice but the number of large follicles was increased (Yoshida *et al.*, 1997). Serine and threonine amino acids are taken up by intracellular proteins (Stocco and Clark, 1993) and phosphorylated by protein kinase A. This phosphorylation may regulate the expression of TGF β like proteins and secretion of ovarian steroids affecting the follicular function. Experiment on Leydig tumour cells showed that treatment with non-phosphorylatable analogues of serine and threonine decreased the expression of P450_{scc} enzyme in mitochondria and therefore progesterone secretion is reduced (Stocco and Clark, 1993).

The phosphorylation of Serine and Threonine within intracellular proteins is important for other intracellular signalling proteins, serving a key role in the activation of intracellular signalling systems. Addition of these amino acids to culture media may stimulate *de novo* production of TGF β like proteins as well as the regulation of steroid production.

Therefore, it was hypothesised that the addition of Serine and Threonine, in the culture medium, may increase the production of steroids and TGF β like peptides and that this may influence follicular growth and atresia.

MATERIAL AND METHODS

Animals

Twenty-one days old prepubertal Quackenbush mice, from the Royal Veterinary College Animal House, were killed according to schedule 1 of Home Office regulations. The ovaries were removed within 1 minute and placed in a petri dish containing supplemented sterile dissection media. The ovaries were cleared from surrounding tissue using watchmaker forceps (Precista 5, 0.3mm, inox, Switzerland) under a dissecting microscope (Leica Wild M28, Milton, Keynes, UK). The media used (for dissection) was Leibovitz L15, (Cat; 413000-021, Life Technologies, Paisley, Scotland) supplemented with 0.005g/ml Bovine Serum Albumin (Cat; 4415555; BDH Biochemical, Poole, England), 25 μ g/ml insulin (Cat; I-9254, Sigma, St Louis, MO, USA), 5 μ g/ml L-Glutamine (Cat; 21051-016, Life Technologies, Paisley, Scotland) and 5 μ g/ml h-transferrin (Cat; T5391, Sigma, St Louis, MO, USA).

Follicle dissection

Follicle dissection was carried out in 2-3 ml of dissection medium, Leibovitz L15 using a 0.33x12mm needle (Cat; AG D-34212, Omnicon, Belgium), in a petri dish (Cat; 627102; Greiner Labortechnik, Gloucestershire, England). The pH of the dissection media was adjusted to 7.35-7.37 and the osmolarity to 275-285 mOsm. The supplemented media (pH 7.35-7.37, 275-285mOsm) was sterilised by filtering through a 0.2µm syringe filter (Cat; 1990-2520, Nalgene, New York, USA) into 12ml sterile tubes (Cat; 184261, Greiner Labortechnik, Gloucestershire, England).

Follicle culture

Preantral follicles, 90-120µm in diameter were cultured in culture medium; α-MEM (Cat; 11900-016, Life Technologies, Paisley, Scotland) supplemented with the same additives as the dissection medium. Preparation of the culture medium was the same as for the dissection medium. Dissected follicles, in 96 well micro plates containing 50µl supplemented culture media, were immediately placed in an incubator containing humidified air at 37°C and 5% CO₂ to protect them from cold stress.

Amino acids

The quantity of amino acids added to the culture medium was calculated based on the amino acid concentrations in mouse plasma (Nagata *et al.*, 1994). L-Serine, (cat; 21101-019, Life technologies, Paisley, Scotland), 0.35g and L-Threonine (Cat; 103053, ICN, Ohio, USA), 0.17g were dissolved in 2ml of hydrochloric acid, 20µl of this was then added to 4 ml α-MEM. A final solution was made by adding 20µl to 1ml of medium to produce concentrations of 165nmol L-Serine and 80nmol L-Threonine in the culture media.

Pregnant Mare Serum Gonadotrophin

5000 iu of powdered PMSG (Cat; 2448; Intervet, Cambridge, England) was dissolved with the solvent provided by the manufacturer and 1ml containing 200iu PMSG was kept as a stock solution and 5µl (1 iu) of this was added to 1 ml incubation medium.

Experimental design

Dissected follicles were randomly divided into 4 groups; a control and three test groups. Control follicles were cultured in supplemented α -MEM. Treated follicles were also cultured in α -MEM supplemented with amino acids, PMSG or a combination of amino acids and PMSG. The PMSG was added at 24h.

Statistical analysis

A two-way analysis of variance was used for data analysis. Data are displayed as means and standard errors of the mean.

RESULTS

Follicle growth, in the culture medium, was slower than expected from published papers (Figure 3.1.1.). Average follicle growth, for the first two days of culture, was 8 μ m/day. Serine and Threonine (AA) were added at beginning of the culture and caused a further increase (1.5 μ m/day) in diameter of cultured follicles over the controls. Addition of PMSG to Serine and Threonine supplemented media did not increase further the diameter of the follicles. Follicles growth faster after the addition of PMSG at 24h. After two days in culture all follicles were atretic.

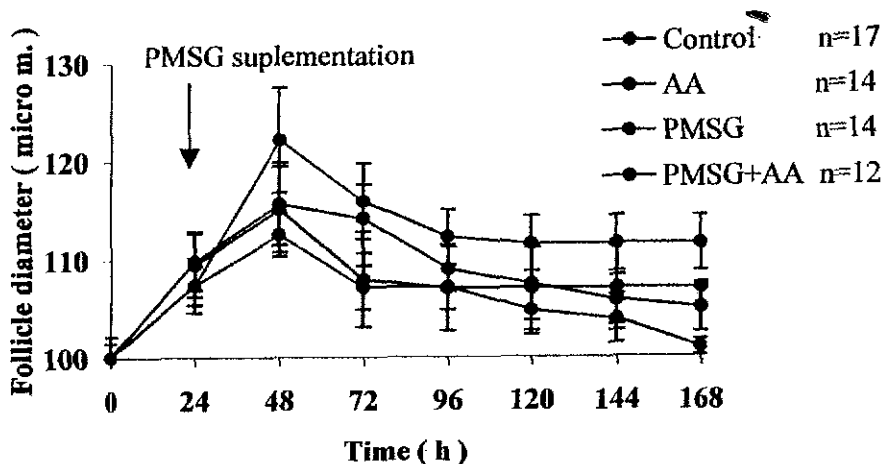


Figure 3.1.1; Pregnant Mare Serum Gonadotrophin was added at 24h and it caused a further increase in the growth of cultured follicles. Amino acids were added at the beginning of the culture and did not significantly increase follicle growth over the control. Amino acids together with PMSG did not cause any significant change in the growth of the follicles.

DISCUSSION

The addition of Serine and Threonine to the culture medium was thought to facilitate *de novo* production of TGF β like peptides that then acted with gonadotrophins to increase mitosis of follicular cells. Addition of Serine and Threonine did not increased follicular growth.

The growth of follicles was poor and not uniform when cultured within a group. When there is contact between the cultured follicles, they fuse into each other and their growth is substantially inhibited (Nayudu and Osborn, 1992). In this experiment, follicles were cultured individually to produce a uniform growth rate.

The addition of PMSG to Serine and Threonine supplemented follicles did not further increase the growth rate of cultured follicles. Preantral follicles grow slowly and they are responsive to gonadotrophins even though they are not dependent on gonadotrophins (McGee *et al.*, 1997). Although gonadotrophins are not essential, their absence results in abnormal folliculogenesis in the mouse ovary. Preantral follicle

survival, cell proliferation and antrum formation are all improved by gonadotrophins. Without gonadotrophins, the growth of mouse follicle is reduced and most become atretic. Therefore, *in vitro*, gonadotrophins are essential for normal development of preantral follicles up to a functional preovulatory stage (Cortvrindt *et al.*, 1997).

The response of follicles to gonadotrophins depends on their size and responsiveness to gonadotrophins increases as the diameter increases (Hartshorne *et al.*, 1994; Mizunuma *et al.*, 1999). The diameter at the beginning of the culture is important in terms of growth. Small follicles require a longer time to enter a rapid growth phase but, large follicles of about 280µm diameter grow quickly (Hartshorne *et al.*, 1994). Follicles with initial diameters of 140-150µm, displayed better growth, in terms of response to gonadotrophins, whereas follicles bigger than 250µm do not since many are already atretic (Nayudu and Osborn, 1992; Mizunuma *et al.*, 1999). Nayudu and Osborn (1992) carried out mouse follicle culture work using the same media used in this experiment. In their work, follicles with initial diameters of 125µm, 150µm and 170µm grew up to 425-450µm after six days of culture and they reported an average growth rate of 60-75µm/day. Boland *et al.*, (1993) and Boland and Gosden, (1994) reported an average growth rate of 30µm/day using similar culture systems to ours and they cultured follicles with initial diameters of 180µm. Smaller mouse follicles, 100-105µm, were cultured by Liu *et al.*, (1998) for four days in a supplemented media (DMEM) and they reported an average growth rate of 10µm/day which was poor when compared with Nayudu and Osborn, (1992), Boland *et al.*, (1993) and Boland and Gosden, (1994). This is probably because the growth of follicles, in culture depends on their initial diameter. Liu *et al.*, (1998) used extremely small follicles and they also used different culture medium that might have effected the growth of the follicles.

We cultured follicles with an average diameter of 100µm and we got an average growth rate of 8µm/day for the first two days of the culture, after which the follicles became atretic. The growth rate of follicles was poor in our culture system because we could not maintain a constant CO₂ tension within the incubator and this may have caused follicles to stop growing. The incubator proved to be faulty and consequently this line of research abandoned.

CHAPTER IV; OVARIAN HISTOLOGY

4.1. THE EFFECT OF SERINE-THREONINE, INJECTED INTO PREPUBERTAL FEMALE MICE, ON FOLLICULAR GROWTH, DIFFERENTIATION AND ATRESIA.

Abstract

Prepubertal female mice, 19-21 days old, were injected with saline (control) or a mixture of Serine and Threonine (test) to determine the effect of these amino acids on follicular growth, differentiation and atresia. The injection of Serine/Threonine (0.2 ml containing 165 nmol L-Serine and 70 nmol L-Threonine in saline) significantly decreased the number of activated primordial follicles entering to primary stage of growth, but after the primary stage, growth rate was increased in the amino acid injected animals. Preantral follicle growth, beyond the secondary stage, was stimulated in the amino acid injected group and more follicles went through to the antral stage. As growth rate increased, the number of atretic follicles was also increased by the amino acid injection. In the early antral stage, the number of atretic follicles, in test animals, was significantly lower, because atresia, in the test group, occurred mostly in the late antral stage. These results show that the injection of amino acids decreased number of follicles entering the primary stage of development and increased the percentage of follicles becoming atretic beyond the primary stage and in late antral stages of development. We suggest that the partial block in follicular growth after the primordial stage and the increase in follicular growth and atresia after the primary stage might be the result of decrease in the expression of TGF β like factors and decreases in the activity of steroidogenic enzymes as well as the decreases in the response of follicles to gonadotrophins. Injection of amino acids probably decreased the expression of TGF β like peptides effecting mitosis and steroidogenic potential of the follicles as well as the expression of receptors for gonadotrophins.

INTRODUCTION

The amino acids, Serine and Threonine are major substrates, for phosphorylation by protein kinases (Alberts *et al.*, 1994; Lodish *et al.*, 1995). Protein kinases covalently add phosphate groups to Serine and/or Threonine on signalling proteins. This phosphorylation is important for the activation of other intracellular proteins involved in gene transcription and signal transduction (Catt, 1996; Grootegoed, 1996). Activation of these proteins facilitates follicular growth and differentiation. Serine/Threonine phosphorylation can modulate the signal transducing ability of TGF- β -like factors as well as their expression within the cell. Under *in vivo* condition, higher doses of TGF- β when injected into rats reduced ovulation rate, but the number of large follicles was increased (Yoshida *et al.*, 1997). Thecal cells, granulosa cells and interstitial cells all express TGF- β like proteins and their expression is regulated by gonadotrophins (Mulheron *et al.*, 1991). Gonadotrophins and oestradiol have been reported to have inhibitory effects on the secretion of TGF- β from rat theca cells *in vitro* (Magofin *et al.*, 1995). Transforming Growth Factor β inhibited thecal androgen production in rat granulosa cells *in vitro* and this led to decreased substrate for aromatization (Magofin *et al.*, 1995). Therefore, TGF- β has both positive and negative actions in hormone-induced differentiation and function of follicular cells. Activin, which is also a member TGF- β superfamily, has been reported to block growth of small preantral follicles in mice and it suppressed positive actions of FSH *in vitro* (Mizunuma *et al.*, 1999).

Injection of Serine/Threonine may modulate the expression of TGF- β like peptides thus effecting the responses of follicular cells to hormones. Therefore, this experiment was designed to test the effect of Serine/Threonine on follicular growth, differentiation and atresia.

MATERIALS AND METHODS

Animals

The experiment used twenty-nine, 19-21 days old mice (QS strain), from the Royal Veterinary College Animal House. The mice were housed in a light and temperature controlled room with free access to water and food. Five to six mice from a litter were kept in the same box and 2 to 3 of them were treated as controls and the other 2 to 3 were treated as the test group.

Experimental procedure

Mice were injected (intra-peritoneal) daily between 10:00 and 12:00, for five days. After five days, the mice were killed by cervical dislocation 60 minutes after the last injection. One ovary from each mouse was kept for histological examination and the other one was used for follicle culture (Chapter 3).

Amino acid solution

The amino acid mixture consisted of 0.35 g L-Serine (Cat; 21101-019, Life Technologies, Paisley, Scotland) and 0.17 g Threonine (Cat; 103053, ICN, Ohio, USA) dissolved in 2 ml HCl, 20 µl of this solution was added in to 4 ml sterile 0.9% saline (pH 7.2). This solution was diluted by taking 32µl and adding to 180µl saline to make a final injection solution of 0.264 µg/200µl L-Serine and 0.132µg/200µl L-Threonine).

Histology

a) Fixation and Embedding

The mice were killed by cervical dislocation, following schedule 1 of the Home Office regulations. The ovaries were removed immediately and placed in Bouin's fixing solution (Cat; 36087 4v, BDH laboratory supplies, Poole, England) for 24h. The ovaries were then washed by placing them for 20 seconds in solutions of 70, 90 and finally 100% alcohol and then stored in 70% alcohol until embedding with wax. For embedding, the ovaries were placed in 90% (1h), 100% (1h), 100% (2h) and 100% (2h) alcohol. Then, the ovaries were placed in a clearing agent, CNP 30 (Cat; CD24, TAAB Laboratories equipment limited, Berkshire, England) and absolute alcohol (1:1)

for 1h and then in clearing agent (CNP30), overnight. The next day, the ovaries were placed in CNP30 and wax (1:1) for 1h at 63°C and then in wax (at 63°C) for a further 1 h before final embedding in wax.

b) Cutting.

Each ovary was serially sectioned using a rotary microtome at 10µm.

c) Staining and counting

Cut sections were mounted on glass slides (Cat; 406/018/04, BDH laboratory supplies, Poole, England) and placed on a warm platform (at 40°C) for drying. When the slides were dry they were stacked in a holder and stained with 210 ml Mayer's Haematoxylin (containing 0.1% Haematoxylin) and 1% Eosin. After staining, the slides were covered with Depex Mounting Medium (Cat; 16125 2B, BDH laboratory supplies, Poole, England) and a coverslip and then left to dry at room temperature.

Every 7th section was counted. Only follicles bearing round oocytes were counted. Atretic and healthy follicles were identified, under the light microscope, at 400X magnification, by counting dark stained nuclei. All follicles were counted and the diameter measured under the light microscope using an ocular micrometer at 100X magnification.

Follicles with an oocyte and surrounded with a part layer of cuboidal granulosa cells were classified as activated-primordial. Follicles with an oocyte surrounded fully by one layer of cuboidal granulosa cells were classified as primary follicles. Follicles with two, three, four layers of granulosa cells were classified as pre-antral. Follicles at the beginning of antrum formation were classified as early antral (Figure 4.1. 1). Follicles with a large fully formed antral cavity were classified as late antral (Figure 4.1 2).

Two sets of slides, from each group of mice were twice counted blind several months apart and the results compared for consistency by paired t-test. There was no difference.

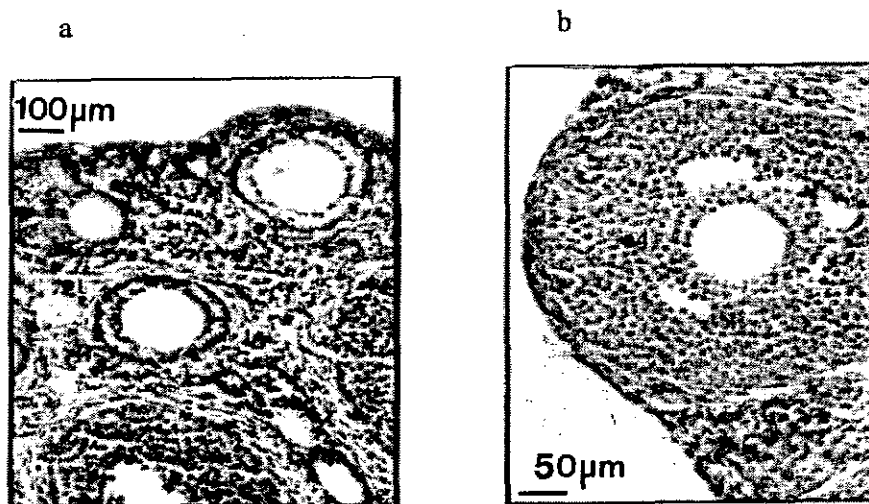


Figure 4.1.1; Photomicrographs of mice ovaries show various classes of follicles at 200X magnification. **a₁:** Follicle in primordial-primary transitional stage surrounded by a few cubical granulosa cells around the oocyte. **a₂:** A primary follicle surrounded by one layer of granulosa cells. **a₃:** A secondary follicle contains two layers of granulosa cells. **b₄:** An healthy early antral follicle, the antrum is not completely formed.

d) Differentiation and Classification of Atretic and non-atretic Follicles.

Cells with condensed chromosomes within the nuclei at X400 magnification were considered atretic due to the its dark apperance. Depending on the number of atretic cells, follicles were classified as early atretic, late atretic or healthy. Follicles with 0-20 atretic cells were accepted as healthy, follicles with 20 -40 atretic cells were considered early atretic and follicles more than 40 atretic cells were considered late atretic.

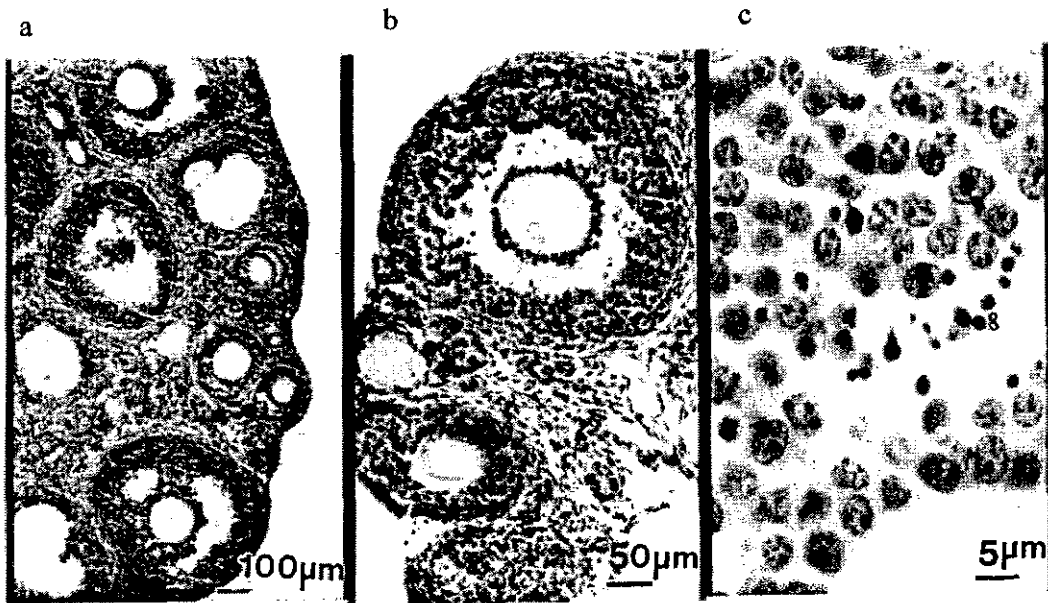


Figure 4.1.2;Three photomicrographs of mouse ovaries with 100X (a), 200X (b) and 400X (c) magnification) showing: **a₁:** Healthy antral follicle. **a₂:** Secondary follicles with two layers of granulosa cells. **a₃:** Primary follicle with one layer of cuboidal granulosa cells. **a₄:** Healthy early antral follicle **b₅:** Atretic antral follicle. **b₆:** Atretic pre-antral follicle. **b₇:** Pre-antral follicle with more than three layers of granulosa cells. **b₈:** Dark stained nucleus indicating atresia.

RESULTS

a) Size distribution

Follicles were classified according to their diameter. The result shows that there tended to be more follicles $>200\mu\text{m}$ in diameter, in mice injected with Serine and Threonine. (Table 4.1.1.). However, the differences were not statistically significant.

Table 4.1. 1; The number of follicles classified according to diameter in saline (Control) or Serine/Threonine (Test) animals (mean $\pm$ sem).

	100-200 μm	200-400 μm	$>400\mu\text{m}$	Total
Control	84 $\pm$ 8.1	77 $\pm$ 11.0	30 $\pm$ 4.6	191 $\pm$ 23.7
Test	86 $\pm$ 6.8	84 $\pm$ 6.0	36 $\pm$ 2.6	206 $\pm$ 15.4

b) Class Distribution

Follicles were also classified according to morphological class. Table 4.1.2 shows that amino acid injection reduced the number of primary follicles ($P<0.05$) and there was no difference in the number of preantral and antral follicles ($P>0.05$). The injection of amino acids significantly reduced the number of primary follicles.

Table 4.1.2; The number of activated primordial, primary, preantral and antral follicles in saline (Control) and Serine/Threonine (Test) injected mice (mean $\pm$ sem).

	Activ. Primordial Follicles	Primary Follicles	Preantral Follicles	Antral Follicles	Total
Control	205 $\pm$ 38.0	65 $\pm$ 6.4	138 $\pm$ 23.1	52 $\pm$ 7.7	460 $\pm$ 81.1
Test	278 $\pm$ 35.7*	46 $\pm$ 5.0*	155 $\pm$ 18.1	57 $\pm$ 6.9	536 $\pm$ 65.7

* $P<0.05$ test compared to control.

Table 4.1.3 also shows that ovaries of Serine and Threonine injected mice tended to contain more antral follicles than the ovaries of control mice. However, the differences were not statistically significant.

Table 4.1.3; The number of antral follicles in mouse ovaries injected with saline or Serine/Threonine (mean $\pm$ sem).

	Antral follicles		
	Early antral	Late antral	Total
Control	28 $\pm$ 4.0	24 $\pm$ 3.7	52 $\pm$ 7.7
Test	31 $\pm$ 3.2	26 $\pm$ 3.7	57 $\pm$ 6.9

c) Atresia

Atresia was not seen in activated primordial or primary follicles from in each group. More preantral follicles, in mice injected with amino acids were atretic ($p < 0.05$) (Table 4.1.4). The numbers of atretic late antral follicles were not different. The numbers of healthy early antral follicles were significantly higher in mice injected with Serine and Threonine (Table 4.1.5). The numbers of healthy preantral and healthy late antral follicles were not different.

4.1.4; The number of atretic pre-antral and antral follicles in mice ovaries injected with saline or Serine/Threonine for 5 days (mean $\pm$ sem).

	Atretic Preantral	Atretic Early antral	Atretic Late antral	Total
Control	45 $\pm$ 7.9	13 $\pm$ 2.5	20 $\pm$ 2.3	78 $\pm$ 12.7
Test	55 $\pm$ 11.5*	8 $\pm$ 2.1*	21 $\pm$ 2.1	84 $\pm$ 15.7

* $p < 0.05$ test compared with control.

Table 4.1. 5; The number of healthy pre-antral and antral follicles in mouse ovaries injected with saline or Serine/Threonine (mean $\pm$ sem).

	Healthy antral follicles			Total
	Healthy preantral	Early antral	Late antral	
Control	93 $\pm$ 15.2	15 $\pm$ 1.5	4 $\pm$ 1.4	112 $\pm$ 18.1
Test	100 $\pm$ 6.6	23 $\pm$ 1.1*	5 $\pm$ 1.6	128 $\pm$ 9.3

* $p < 0.05$ test compared to control.

DISCUSSION

The numbers of activated primordial follicle in the ovaries of mice injected with Serine /Threonine were not different from control mice ($p > 0.05$). In the test group, the number of follicles at the primary stage was significantly reduced suggesting that the progression of primordial stage to the primary stage was significantly delayed by the injection of Serine and Threonine. The mechanisms that induce primordial follicles to enter the growth phase are not known but, one possibility might be reduced signalling activity or synthesis of Transforming Growth Factor beta (TGF- β). This growth factor is generally required for initiation of DNA synthesis and is synthesised within the ovary (Dorrington *et al.*, 1993). Another possibility is that the transition of primary follicles to preantral and early antral stage has been stimulated in test mice, but that is not possible because there was no differences, in the numbers of healthy preantral and early antral follicles between the groups.

Injection of Serine/Threonine may also decrease endogenous production of TGF- β by decreasing protein kinase-C activity within the theca cells. Growth of primordial follicles is affected by the oocyte derived growth factor, growth and differentiation Factor -9 (GDF-9) which is also a member of the TGF- β family facilitating early follicular growth in mice (McGrath *et al.*, 1995). Injection of Serine/Threonine may also decrease the production of this growth factor. Follicles after the primary stage were not affected by reduced production of GDF-9 (Dong *et al.*, 1996). This growth factor is synthesised within the oocyte of primary follicles. It has been reported that, in GDF-9 deficient female mice, the number of primordial and

primary follicles was unchanged, but that there was a block in follicular development, beyond the one cell layer follicle stage (Dong *et al.*, 1996).

It is generally accepted gonadotrophins are not necessary for the initiation of primordial follicle growth (Eppig and O'Brien, 1996). The development of mouse primordial follicles *in vitro* to the secondary stage, can be achieved without gonadotrophins (Boland *et al.*, 1993; Eppig and O'Brien, 1996). The later stages of follicular growth and differentiation (beyond the committed stage, follicles with 3 layers of granulosa cells) are affected by pituitary gonadotrophins (Scaramuzzi *et al.*, 1993). This has been confirmed by experiments with FSH knockout mice. The injection of Serine and Threonine may increase gonadotrophin secretion facilitating follicle growth after primary stage, but number of preantral and antral follicles were not different (Table 4.1.2) suggesting that follicle growth after the primary stage was not effected by Serine/Threonine injections.

Reduced atresia in amino acid injected mice also can be a result of increase in LH response of granulosa cell due to the expression of LH receptors on granulosa or increases LH and FSH secretion because of expression of TGF- β . Late follicular differentiation is substantially effected by pituitary gonadotrophins. In culture, FSH did not effect the mean number of granulosa cells, but it increased follicular metabolism, in terms of lactate and oestradiol production (Boland *et al.*, 1993). The addition of LH further stimulated lactate production, but caused a significant decrease in oestradiol production (Boland *et al.*, 1993).

In the rat, TGF- β potentiated luteinizing hormone receptor expression on granulosa cells Gitay-Goren *et al.*, 1993). Treatment of oestrogen primed rat granulosa cells, *in vitro*, with TGF β , for 48 h, increased FSH receptor mRNA levels (Dunkel *et al.*, 1994). In FSH- β subunit deficient mice, folliculogenesis beyond the antral stage is blocked and as a result, these females were infertile (Kumar *et al.*, 1997). The number of atretic pre-antral follicles increased in test mice. This might be a result of decreased the signalling ability of TGF- β because of Serine and Threonine injection. Transforming Growth Factor Beta signals through a Serine/Threonine kinase receptor. In Serine/Threonine kinase receptor deficient mice, FSH and LH secretion was suppressed (May *et al.*, 1995; Matzuk *et al.*, 1995) this may be a result

of a changed negative feedback effect of ovarian steroids. Decreases in gonadotrophin secretion will also increase atresia in preantral follicles just before the antral stage.

Atresia in early antral stage was significantly low in Serine and Threonine injected mice. This might be due to the decrease in expression of TGF- β or it might be the result of changes in steroidogenic enzyme activity within the ovary. It is also possible that reduction in atresia due to the increase in gonadotrophin responses of follicular cells as a result of changes in the expression of TGF- β like proteins within the ovary, which in turn can cause an ovarian feedback effect on hypothalamus affecting gonadotrophin secretion.

Treatment of immature rat thecal-interstitial cell with TGF β decreased androgen production while it enhanced progesterone production (Fournet *et al.*, 1996). Increased progesterone production indicates that activity of P450_{scc} enzymes increased. Experiments on rat thecal-interstitial cell showed that, TGF β alone or with LH increased P450_{scc} enzyme activity but, did not alter P450_{arom} activity (Magoffin *et al.*, 1989) and TGF β has no effect on oestrogen production from rat granulosa cells (Ying *et al.*, 1986). Increased progesterone production suggests an atretic effect of TGF β because progesterone has luteotrophic effect on follicular cell. More work is required to find out if there are any changes in the expression of TGF β like proteins and steroidogenic enzymes within the ovary (chapter 9).

The number of antral follicles was not changed by Serine/Threonine injection indicating that Serine and Threonine injection did not alter the secretion of FSH and the response of the follicles to FSH. Formation of an antrum is effected by FSH. Further work is required to investigate the effects of Serine/Threonine injection on gonadotrophin secretion and on gonadotrophin receptor expression.

These results show that injection of amino acids significantly reduced follicle growth before the primary stage and increased the incidence of atresia in preantral and antral stage follicles. The injection of serine and threonine may have reduced the expression of TGF- β and this may then have affected follicle growth (see chapter VIII).

CHAPTER V; TESTIS HISTOLOGY

5.1. THE EFFECT OF INJECTING A MIXTURE OF SERINE AND THREONINE ON TESTICULAR FUNCTION

Abstract

Prepubertal male mice (QS strain) were injected for 5 days with saline (control) or a mixture of Serine and Threonine to test the effects on testicular function. The testes were dehydrated and serially sectioned at 10 μ m and then stained with PAS (Periodic Acid Schiff). The diameter of tubules, diameter of Leydig cells and the numbers of spermatids were measured on very 80th section of the testis. The number of stage 2-3 spermatids was decreased by Serine and Threonine injection ($P<0.05$). The total numbers of spermatids per testis for control and test groups were estimated as 1.66 ± 0.32 and 1.06 ± 0.05 ($\times 10^6/\text{mm}^3$) respectively. This experiment shows that Serine and Threonine injection reduced the number of spermatids while the diameters of Leydig cells and the diameters of tubules were not affected. The reason for reduced number of spermatids might be a result of increased expression of TGF- β s which increased apoptosis of the spermatogonia and that probaly resulted with reduced number of 2-3 stage spermatids.

INTRODUCTION

The aim of this work was to measure the effect of the injection of Serine and Threonine on spermatogenetic function in terms of changes in tubule diameter, diameter of Leydig cells and the number of spermatids in the mouse testis because of the differences in expression of TGF- β s due to the injection. The expression of TGF- β s can affect mitosis of cells affecting hormone (testosterone) secretion and spermatogenesis.

Cellular development and differentiation are regulated by Serine and/or Threonine phosphorylation of intracellular proteins effecting cellular proliferation and differentiation (Alberts *et al.*, 1994; Lodish *et al.*, 1995, Catt, 1996 and Grootegoed, 1996). Serine and Threonine amino acids are substituted within the intracellular proteins and phosphorylated by protein kinase A. This phosphorylation is important for testosterone production (Stocco and Clark, 1993). Therefore, Serine and Threonine phosphorylation of intracellular proteins may influence spermatogenic potential within the testis due to the changes in steroid secretion and the production of new proteins, such as TGF β_1 , β_2 and inhibins which have local inhibitory effects on spermatogenesis (Olaso *et al.*, 1998). TGF β_1 and β_2 have been detected in foetal and neonatal rat testes and their receptors are present in developing rat testes from fetal day 13.5 to postnatal day 3. The first expression of receptors for TGF β_1 and β_2 , in Leydig cells is from fetal day 16.5, but they were not detected in Sertoli cells.

Under *in vitro* conditions, 10 ng/ml of TGF β_1 and β_2 significantly reduced the number of gonocytes (spermatogonium) after two days in culture (Olaso *et al.*, 1998). They concluded that, TGF β and β_2 directly increased apoptosis without changing mitotic activity and higher amounts of TGF β_1 and β_2 in Leydig cells during foetal life have an autocrine effect decreasing testosterone production during foetal development.

It was hypothesised that Serine and Threonine might modulate steroid production and the expression of TGF- β like factors and this in turn affect function of the testes.

MATERIAL AND METHODS

Animal and injection

The animals and the preparation of the amino acids for injections were the same as reported in chapter, 3 and 4

Design of experiment

Sixty minutes after the last injection the mice were killed (according Act 1 of Home Office regulations). Just before killing, the mice were weighted to see if there was any effect of the injections on body weight.

Removal of testis, its fixation and embedding

The mice were killed by cervical dislocation and their testes taken out, using fine forceps and placed in Bouin's fixing solution (Cat; 36087 4v, BDH laboratory supplies, Poole, England) for 24h. The testes were dehydrated and embedded in wax as reported (Chapter 4).

Cutting and staining

Each testis was serially sectioned at 10µm, using a rotary microtome and sections placed on glass slides (Cat; 406/018/04, BDH laboratory supplies, Poole, England). The sections were de-waxed by placing them in a clearing reagent CMP30 (Cat; CD24, TAAB laboratory supplies, Berkshire, England) for 7 min, rehydrated by placing them in graded alcohol from 100% to 90, 50 and 30% alcohol each for 1 min and finally in distilled water for 2 min. After rehydration the sections were placed in periodic acid (Cat 104324Q; BDH laboratory supplies, Poole, England) for 5 min, washed several times with distilled water and then washed in running tap water. The sections were placed in Schiff's solution (Cat 19120 3S; BDH laboratory supplies, Poole, England) for 30 min. and washed in tap water for 8 min, placed in iron Haematoxylin, washed for 7 min in tap water, the sections were placed in acid alcohol (1 ml HCl + 100 ml 70% ethyl alcohol) for 1 min, washed in distilled water and dehydrated again. Finally the sections were placed in clearing reagent CMP 30 for 7 min, mounted with Depex Mounting Medium (Cat; 16125 2B, BDH laboratory supplies, Poole, England) and coverslipped.

Measurements and Calculations

Diameter of tubules

Only the diameters of round tubules, with a good circular appearance were measured. Diameters were measured vertically and horizontally and the mean calculated. Only tubules whose ratio of horizontal and vertical diameters fell between 0.9 - 1.1 were included (Figure 5.1.1c). Diameters were measured under the light microscope as before (Chapter 4) using an ocular micrometer at 10 x 10 magnification. Round tubules were measured in every 80th section. In every section, 5 - 20 tubules were measured.

Diameter of Leydig cell nucleus

In every 80th section, vertical and horizontal diameters of 50 cells were measured under an oil-immersion lens at 100x10 magnification (Figure 5.1.1b). Two set of slides, from each group, were counted blind, twice and the results compared for consistency by t-test. There were no statistically significant differences.

Number of spermatids

Spermatids at stage 2-3 of the spermatogenetic cycle were counted. Differentiation of these spermatids was detected by looking at acrosomal granules and their position and size around the nucleus, which was quite clear with the red PAS stain (Picture 5.1.1a).

Measurement of testis volume

The total number of sections for each testis was recorded. The mean area of every 80th section was measured using image analysis computer software (ZEISS KS 300, version 3, copyright 1997). The computer was connected to a light microscope (Olympus BH-2, Seri no; 238956, Japan) with a colour video camera (Model; KY-F55BE). Only testis was measured, the epididymis was not measured. **In saline treated group, two mice were not included because they were not mature.**

Total number of sections for each testis	= TNS
Mean area of every 80th section	= MA80 (mm ²)
Section thickness	=ST (μm)
Testis volume in wax (TV)	= TNS x ST x MA80 (mm ³)

Calculations

Calculations were done according to the formulas by Vergouwen *et al.*, (1995).

Number spermatids.

The number of spermatids was calculated using the following formula:

ST= Section thickness (μm)

TV= Testis volume in wax (mm³)

Q= Number of nuclei

A= Area counted (mm²)

Ns = Number of spermatids per testis

Ns=Q/AxSTxTV (Vergouwen *et al.*, 1995).

Statistical analysis

Data were analysed by using an unpaired T- test.

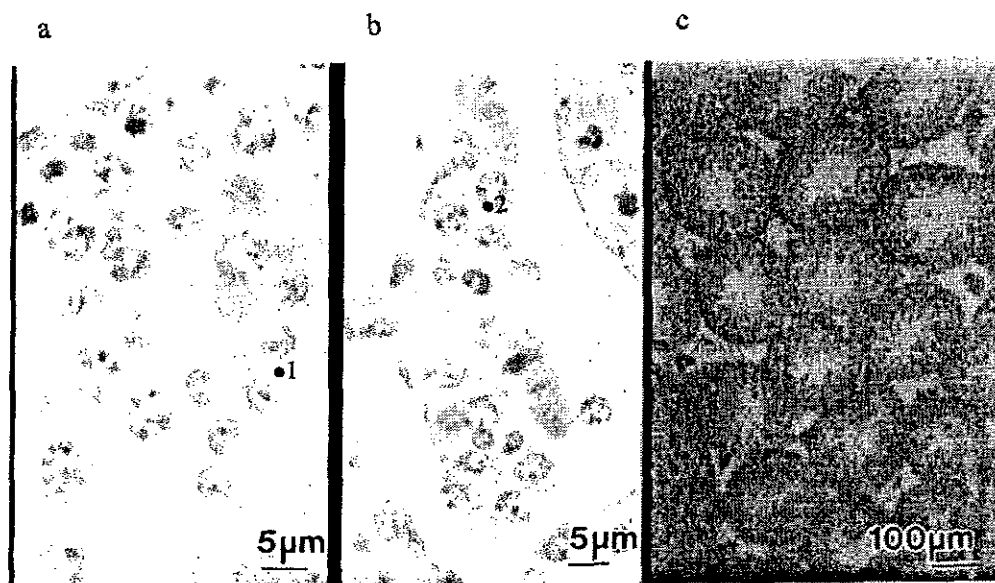


Figure 5. 1. 1; A microphotographic illustration of mouse testes showing tubules (c), Leydig cells (b) and spermatids (a). Stage 2-3 spermatids (on PAS stained sections) were differentiated by size and colour of acrozoal granules. After staining acrozoal granule can be differentiated by the red colour around the nucleus (a1). Leydig cell are generally found in groups in intertubular area (b2).

RESULTS

The diameters of the tubules, the diameters of the Leydig cell were not significantly changed by the injection of Serine and Threonine (Figure 5.1.2 and 5.1.4). Injection of Serine and Threonine significantly decreased number of 2-3 stage spermatids (Figure 5.1.3.)

Differences in tubular diameter of saline and amino acid injected mice

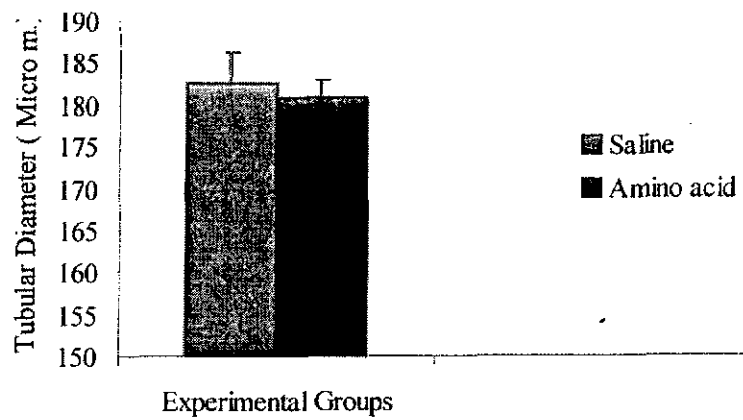


Figure 5.1.2; Amino acid injection did not increased tubule diameter ($t= 0.48$; $P = 0.6378$).

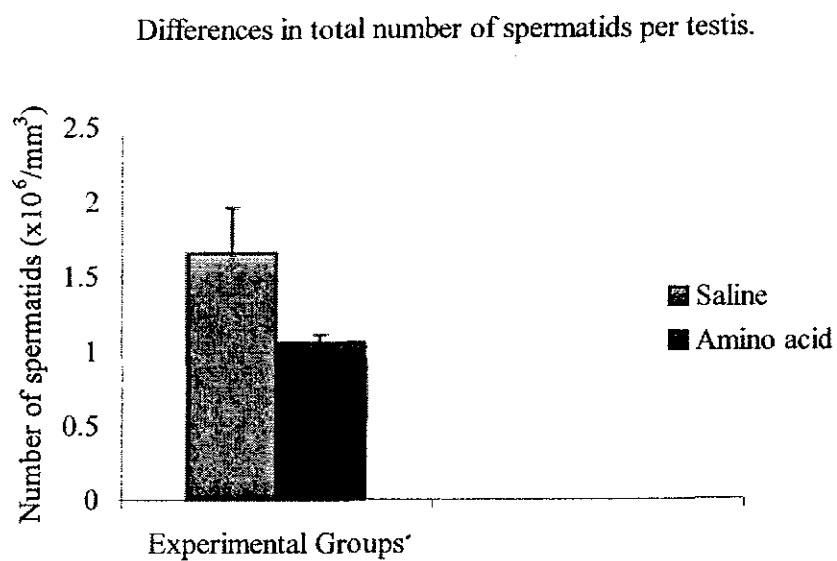


Figure 5.1.3; Amino acid injection decreased the number of stage 2-3 spermatids in each testis ($t=1.96$; $P= 0.0714$).

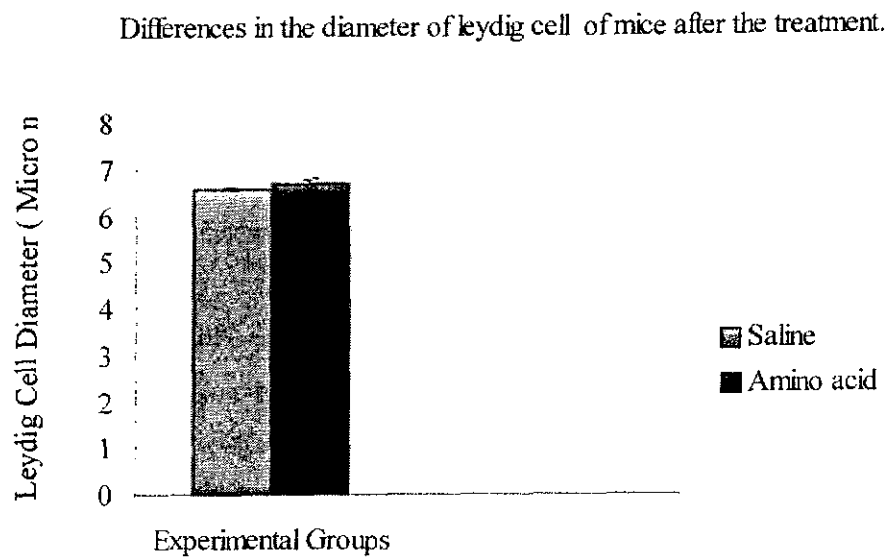


Figure 5.1.4; Diameter of Leydig cells was not increased in amino acid injected animals ($t = -1.19$; $P = 0.2547$).

DISCUSSION

Increase in tubular diameters is used an indirect measure of spermatogenic function (Berndtson and Jones, 1989), because, increased surface area of the spermatogenic epithelium can support more Sertoli cells and spermatogonia. Increased surface area of the basement membrane, is generally related to higher testosterone production (Berndtson and Jones, 1989). High testosterone production has been reported to have a negative effect on hypothalamus resulting with a decrease in gonadotrophin secretion and therefore sperm production. Studies using high doses of testosterone injected (weekly, 200mg) in normal man, resulted in azospermia. This negative effect of high doses of testosterone on sperm production was thought to be a result of negative feedback effect of testosterone on gonadotrophin secretion and this was confirmed by hormone analysis in man which showed that, plasma concentrations of testosterone and oestradiol increased by 2.5-fold whereas plasma concentrations of LH and FSH decreased (Anderson and Wu, 1996; Bebb *et al.*, 1996).

The injection of amino acids did not increase in tubular diameter and also the diameter of Leydig cells did not changed. Increased Leydig cell diameter can be related to increased secretion of testosterone. According to this results we can not say that the decreased number of spermatids in amino acid injected mice caused by testosterone while diameter of the tubules and Leydig cells did not changed. Low number of spermatids, in amino acid injected mice, might be caused by the changes in TGF- β expression. It was reported that endogenous TGF- β increase the apoptosis of genocytes and this lead to a decrease in sperm production (Olaso *et al.*, 1998).

This results show that number of spermatids was decreased by Serine and Threonine injection. Amino acid injection may increase the expression of TGF- β like factors within the testis leading to apoptosis of spermatogonium and consequently decrease in the number of spermatids.

CHAPTER VI; AMINO ACID EFFECT ON LH SECRETION

6.1. THE ACUTE EFFECT OF SERINE AND THREONINE ON PLASMA CONCENTRATIONS OF LH IN ANOESTRIOUS EWES TREATED WITH GnRH OR A GnRH AGONIST, AZAGLY-NAFARELIN.

Abstract

Three experiments were conducted using 16 anoestrous ewes to determine the effects of a mixture of Serine and Threonine on the secretion of LH induced by GnRH or an analogue of GnRH (A-N; Azagly-nafarelin; Intervet International BV, Boxmeer, The Netherlands). In the first experiment, the ewes were injected intravenously with amino acids (2.0 g of L-Serine and 1.0g L-Threonine in 20 ml saline; AA) or saline (S) with or without GnRH 1h and 5h after the start of sampling. The experiment was replicated twice to give the following treatments (I) S/S (n=8), (II) AA/GnRH (n=7); (III) S/GnRH (n=6), (IV) S/AA+GnRH (n=5) and (V) AA/AA+GnRH (n=6). Blood samples were collected every 15 min for 7h. In the second experiment, 7 sheep were given an injection of saline and 8 sheep were given an injection of amino acid mixture as above. Injection were given every two hours starting at 10:00 and continuing until 18:00 and blood sampling starting at 10:00 until 19:00.

In the third experiment, 7 treatments were used over 16 days in a randomised latin square design, so that each ewe received each treatment. The treatments were saline, GnRH (0.1, 1 and 10 ng) or A-N (0.001, 0.01, 0.1 and 1 ng), injected intravenously in 2ml saline. Blood samples were collected at intervals of 15, 30 or 60 min from 30 min before until 240 min after injection. In all three experiments jugular venous blood was collected from an indwelling jugular venous catheter, centrifuged at 2900 RPM at 4°C and stored at -20°C.

In experiment one, injection of amino acids before and together with GnRH caused a significant increase in LH secretion. Injection of sheep with saline followed by GnRH or GnRH +AA caused significant increases in LH secretion, the differences in LH response between GnRH and GnRH+AA were not significant. Peak concentrations of

LH for saline-saline; Saline-GnRH; saline-AA/GnRH; AA-GnRH and AA-AA/GnRH were 0.54 ± 0.07 ; 1.34 ± 0.35 ; 1.40 ± 0.44 ; 3.50 ± 0.99 and 4.16 ± 0.73 respectively. In the second experiment, the injection of amino acids increased LH secretion, but not significantly. In the third experiment, small doses of GnRH or GnRH analogue were not as effective in inducing an LH pulse, whereas higher doses caused large increases in LH secretion. The analogue was 38 times more potent than GnRH. Peak levels of LH for 0.1, 1, 10 ng GnRH; 0.001, 0.01, 0.1 and 1 ng analogue were 2.04 ± 0.48 , 5.01 ± 0.83 , 12.55 ± 2.66 ; 0.62 ± 0.09 , 0.78 ± 0.14 , 6.60 ± 1.80 and 46.21 ± 2.66 respectively. These results indicate that injection of amino acid influenced LH secretion. They also show that azagly nafareline is a potent GnRH analogue. The effect of these amino acids on LH secretion is probably mediated by ovarian feedback mechanisms influencing the response of gonadotrophs to GnRH or by an effect on hypothalamus, increasing the secretion of GnRH.

INTRODUCTION

This experiment was designed to determine if Serine/Threonine injections were capable of altering hypothalamo- pituitary function and effecting LH secretion. The identification of the mode of action of Serine and Threonine on ovarian function also requires an understanding of their effect on hypothalamo-pituitary function that might indirectly influence follicular dynamics. Nutritional stimulation of gonadotrophin secretion has been reported to be accompanied by changes in the concentration of metabolic substances such as glucose, insulin and amino acids in the peripheral circulation. It also has been reported that short periods (6h) of infusion of prepubertal ewe lambs with 15-30g L-Arginine was capable of altering LH pulse frequency (Recabarren *et al.*, 1996). The increases in LH pulse frequency during arginine infusion were though to be a result of an action stimulating hypothalamic GnRH release. It is unlikely that very much hypothalamic GnRH reaches the ovary because of dilution in the peripheral circulation and because of its rapid degradation by enzymes. GnRH analogues exhibit a higher resistance to degradation. Therefore, they are generally used to stimulate ovarian function (Tsafiriri and Adashi, 1994).

Early follicular growth is not dependent on pituitary gonadotrophins, but a basal level is required for follicular maintenance and survival. As follicular development progresses, follicles become increasingly gonadotrophin-dependent, especially for FSH.

Secretion of FSH and LH influences the final stages of follicular growth and development. Suppression of pituitary gonadotrophins by immuno-neutralization or by chronic treatment with GnRH agonists decreases pre-ovulatory follicle growth beyond 2.5 mm. Pulsatile secretion of GnRH into the hypophyseal portal circulation controls pituitary LH release. Short-lived pulses of GnRH produce corresponding pulses of LH (Clarke, 1996). When ewes were immunised against GnRH, in the prepubertal stage or around puberty plasma LH and FSH concentration were decreased for up to 2 years (Brown *et al.*, 1995).

After the suppression of gonadotrophins by immunisation, the infusion of FSH and non-pulsatile LH induced follicular development but infusion of LH alone, in a pulsatile manner, after the down regulation with the GnRH analogue, buserelin, did not induce preovulatory follicle growth. Therefore, FSH is the principal gonadotrophin controlling the number of growing preovulatory follicles, follicular development and differentiation thereby controlling the number of large antral follicles destined to ovulate (McNeilly, 1991; Wang and Greenwald, 1993).

MATERIAL AND METHODS

Animal

Sixteen cross bred sheep (Suffolk and Cheviot, 2-6 years old), with an average weight of 63 kg were purchased from a commercial company. From the 10 of Jun. 1997, they were housed indoor and were penned together in groups of 3, 2 or 4 and fed a diet of *ad libitum* hay, water was freely available.

Injection of GnRH

Human synthetic GnRH was purchased from Sigma chemical company (Cat; L-7134, St Louis, MO, USA). On the 19th and 21st of June, 150 ng of GnRH was injected once a day.

Preparation and injection of amino acids.

63.96g L-Serine (Cat no; 21101-019; Life technologies, Paisley, Scotland) and 31.98 g L-Threonine (Cat; 103053, ICN, Ohio, USA) were dissolved in 640 ml saline (pH 7.4). The solution was sterilised by filtration then 20 ml was injected through the jugular catheter. Therefore, each treated sheep was injected with 2g L-Serine and 1 g L-Threonine. The amino acids were injected once a day, on the 19 and 21 of Jun. 1997. Eight sheep injected with amino acids and the other 8 with saline acting as controls.

Experimental design

Experiment 1

The sheep (n= 16) were divided in groups (according to body weight) and injected intravenously, twice (at 11:00 and at 14:00) with 20 ml combinations of saline, L-Serine (0.1g/ml; Cat No 21101-019, Life Technologies, Paisley, Scotland) and L-Threonine (0.05g/ml; Cat No 103053, ICN, Ohio, USA) and 150 ng GnRH (Cat No L-7134, Sigma, St Louis, MO, USA) in 2 ml of saline, as shown in table 1. Blood samples (4 ml) were taken at 15min intervals.

Table 6.1. 1; Experimental design

Group	Number of sheep	Injection time	
		11:00	14:00
1	8	S	S
2	6	S	GnRH
3	5	S	AA+GnRH
4	7	AA	GnRH
5	6	AA	AA+GnRH

AA=Amino acid

S=Saline

GnRH= Gonadotrophin Releasing Hormone

Experiment 2

The ewes were divided into groups and injected with either saline (Control, n=7) or a mixture of Serine and Threonine (test, n=8), using the same dose as used in test1, every two hours from 10:00 for a total of 5 injections. Blood samples (4 ml) were taken via a jugular venous catheter for 9 hours at 20 min intervals starting at 10:00.

Experiment 3

The ewes were injected with saline (Control, n= 14) or increasing doses of GnRH (0.1, 1 and 10µg; n=13, n=13 and n=14 respectively) or a GnRH analogue, azagly nafarelin (1,10, 100 and 1000 ng; n=13, n=13, n=14 and n=14) respectively. Blood samples (4 ml) were taken at intervals ranging from 15-60 minutes, via a jugular venous catheter for 6 hours starting at 11:00. On the 14th, 16th, 18th, 20th, 23th, 25th and 27th. Some of the catheters malfunctioned and the saline injected control group, sheep 3, 4 and 6 were not bled. Thirteen sheep were injected with 0.1 ng GnRH and in this group sheep 6 was not bled. In the 1 ng GnRH, sheep 7 was not bled and in the 0.001, 0.01, 0.1 and 1 ng GnRH analogue groups, sheep 3, 10, 5 and 3 respectively were not bled. These animals were excluded.

LH Assay

Label

Na¹²⁵I was obtained from Mr Michael Avella, Royal Veterinary College, UK. Ovine, LH, for iodination, was obtained from NIDDK (Cat; AFP7071B, Beltsville, USA). This protein was iodinated by using the Chloramine-T method (Hunter and Greenwood, 1970).

Iodination procedure

Column preparation

A Sephadex G-50 Column was prepared by adding 1g sephadex G-50 (Cat; 195580, ICN Biomedical, Ohio, USA) to 50 ml de-ionised water and left overnight at 4°C to swell. The swollen sephadex was poured into a column constructed from

30x12mm-glass tubing sealed at one end with 50 μ m cotton gauze, 2mm metal gauze and a rubber stopper. A blunted 19G needle was inserted through the stopper and attached to 1.0mm ID polyvinyl tubing. Part of the tubing was formed into 5-7 loops of a tight coil which was placed near a counter for monitoring the level of radiation flowing from the column. The end of the tubing was held in a clamp. The Sephadex was poured into the column as gently as possible to avoid any air bubble forming.

Equilibration of the column

The column was equilibrated with 0.05 M phosphate buffer and with 0.25% BSA (Cat; 6793, Sigma, St Louis, MO, USA). This buffer was made up by adding:

1.23 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Cat; 301324Q, BDH, Poole, England)

2.68g Na_2HPO_4 (Cat; 102494C, BDH, Poole, England) in 500ml de-ionised water, pH 7.5 and then 1.25g BSA (Cat; A-6793, Sigma, St Louis, MO, USA) was added.

Preparation of protein

Five μ g of oLH placed in 5 μ l of the 0.25 M phosphate buffer. This buffer was prepared by adding the following;

7.4 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Cat; 301324Q, BDH, Poole, England)

26.8g. Na_2HPO_4 (Cat; 102494C, BDH, Poole, England) and made up to 1L with de-ionised water, pH 7.5. The LH was stored in a freezer at -20 until the day of iodination.

Preparation of Chloramine T

0.1g Chloramine-T (Cat; 276704E, BDH, Poole, England) was added in 20 ml of 0.05M phosphate buffer. This buffer prepared by adding:

1.23 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Cat; 301324Q, BDH, Poole, England)

2.68 g. Na_2HPO_4 (Cat; 102494C, BDH, Poole, England) in 500ml de-ionised water, pH 7.5.

Preparation of Sodium metabisulphite

0.06 g sodium metabisulphite (Cat; 10356, Analar (BDH), Poole, England) added in 20 ml of 0.05 M phosphate buffer, pH 7.5.

Preparation of Potassium iodide

0.25g potassium iodide added in 25 ml of 0.05 M phosphate buffer, pH 7.5, containing 0.1% BSA.

Reaction

To 5 µg oLH in a glass reaction tube, 25µl 0.05M phosphate buffer, 5µl radioactive iodide (18.5MBq) was added and mixed by gentle shaking, the reaction was started by adding 20µl Chloramine-T and continued for 30-60 seconds and terminated by adding 100µl sodium metabisulphite. To increase the volume, 200µl potassium iodide (Cat; 22,194-5, Aldrich, Steinheim, Germany) was added and the contents transferred to the column by glass pipette. The end of polyvinyl tubing was opened and the liquid on the top of gel allowed to run down into the gel. The reaction tube was washed with 200µl potassium iodide and transferred to a column and then the clamp was opened and the liquid collected in a glass container.

Elution and collection of fractions

The column was eluted by washing with 0.05M phosphate buffer, pH 7.5, containing 0.1% BSA. Radioactivity was monitored by using a gamma counter. Approximately, 1ml fractions were collected in glass tubes. The activity of each tube was monitored by placing 2 µl of each fraction in a glass tube and counting for 1 minute in a gamma counter (Packard, Cobra-II, Auto-Gamma, Meriden, USA), a technical result is displayed on figure 6.1.1.

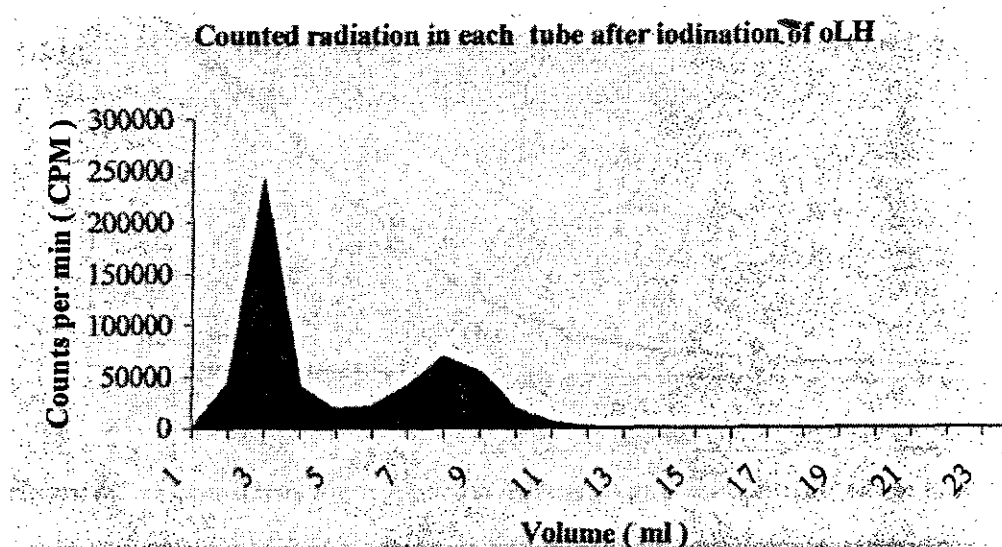


Figure 6.1.1; After iodination of LH, 24 fractions (each fraction of approximately 1ml) were collected and 2 μ l of each fraction counted in a gamma counter. The first fraction of iodination product was used for the measurement of LH.

Reference preparation

Ovine LH for use as the reference preparation obtained from NIDDK-NIH, oLH-26 (Cat; APF192279, Beltsville, USA). Standards were prepared in concentrations of 0.2 ng/ml to 100 ng/ml.

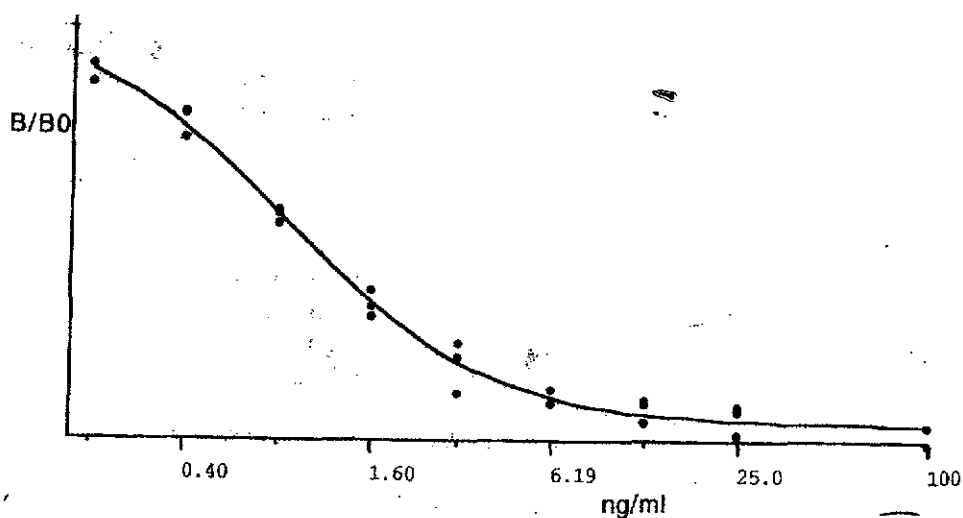


Figure 6.1.2; Standards made up from 100ng/ml and serially diluted to 0.2ng/ml.

Measurement of Plasma LH level

Plasma LH concentrations were measured by RIA. The antibody for LH was developed in a rabbit (R 29) and kindly provided by Dr BK Campbell (University of Edinburgh, Centre for Reproductive Biology, Edinburgh. Rabbit IgG (Cat; S0022-220) and Normal Rabbit Serum (Cat; S030-220) was obtained from Scottish Antibody Production Unit, Low Hospital Carlisle, Lanarkshire, Scotland.

Table 6.1.2; Procedures applied in LH assay

Tubes	Buffer	Sample	AB1	Left 48h at 4°C	Label	Left 48h at 4	AB2 +NRS
Standard 1	200	100	100		100		200
Standard 2	-	-	-		100		-
NSB	300	100	-		100		200
B ₀	200	100	100		100		200
Unknown	200	100	100		100		200

QC=Quality controls NSB= Non specific binding AB1= First antibody
AB2= Second antibody NRS= Normal rabbit serum

100µl of unknown plasma was placed in assay tubes in duplicate with 200µl of assay buffer and 100µl of the first antibody (R29) and incubated for 2 days at 4 °C, 100 µl label was then added giving 12000-15000 CPM and the tube incubated at the same temperature for another 2 days (Table 6.1.2). The second antibody and normal rabbit serum were then added and incubated for 24h then 1 ml 0.01 M phosphate buffered saline (PBS) was added and assay tubes immediately centrifuged, at 2900 rpm for 1h, decanted and counted in a gamma counter. The amount of LH was calculated by using computer software, Assay Zap Version 2.32, Universal assay calculator, Copyright P. L. Taylor 1987-1992, Published and distributed by Biosoft 22 Hills Road, Cambridge CB2 1JP, UK).

Assay buffer was prepared by dissolving the following reagents

1.75g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Cat; 301324Q, BDH, Poole, England)

5.90g Na_2HPO_4 (anhydrous) (Cat; 102494C, BDH, Poole, England)

45g NaCl (Cat; 10241-5K, BDH, Poole, England)

5g BSA (Fraction 4) (Sigma, A-6793, St Louis, MO, USA)

in 5L de-ionised water, pH 7.5. To 1 L of this buffer, 18.62g Ethylenediaminetetraacetic acid (EDTA, disodium salt) (Sigma, Cat E-4884, St Louis, MO, USA) was added and the pH adjusted to 7.5 used for preparation of label.

Assay parameters

Assay parameters were calculated from three oLH assays. Sensitivity is a minimum amount of hormone that can be detected. Sensitivity of the LH assay was measured by calculating the value of B_0 (assay zero) at 95% confidence interval then determining the amount of LH matching the value calculated. The sensitivity of LH assay was 0.22 ± 0.01 ng/ml. Intra and inter assay coefficient of variations were calculated for each quality control at high, medium and low concentration of LH. Intra assay coefficient of variation was calculated as the coefficient of variation of high, medium and low quality controls. Inter assay coefficient of variation was calculated by taking mean standard deviation of quality controls for 3 assays, dividing by their mean then multiplying by 100. Intra-assay coefficient of variation for high, medium and low were 10.5, 11.1 and 11.4. Inter-assay coefficient of variation for high, medium and low were 20.2, 10.5 and 16.2 respectively.

Statistical analysis

Data were analysed by using a randomised block design (Anova). Total variation, variation between groups, variation between blocks and variation within groups were calculated. Variation between groups was divided by variation within groups to find the F value. When F value was larger than the value at the 0.05 probability level, groups were considered as significantly different from each other. To identify which samples are significantly different, at any time of sampling, between the groups, Last Significant Difference (LSD) method was used. Data are displayed on the figures as mean $\pm$ sem.

RESULTS

EXPERIMENT 1

Injection of GnRH significantly increased LH secretion ($P < 0.05$). Peak LH levels in plasma of sheep injected twice with saline-saline (at 10:00 and at 14:00), saline-GnRH and Saline- Serine/Threonine together with GnRH (S-AA/GnRH) were 0.54 ± 0.07 ; 1.34 ± 0.35 and 1.4 ± 0.44 respectively . There was no difference after the second injection when the sheep injected with GnRH or amino acid with GnRH (Figure 6.1.3). LH secretion increased significantly when the sheep were injected first with amino acid and second with GnRH alone. The injection of amino acid before or together with GnRH also increased GnRH induced LH secretion. The increase tended to be higher when the sheep were injected first with amino acid and second with amino acid and GnRH (Figure 6.1. 4).

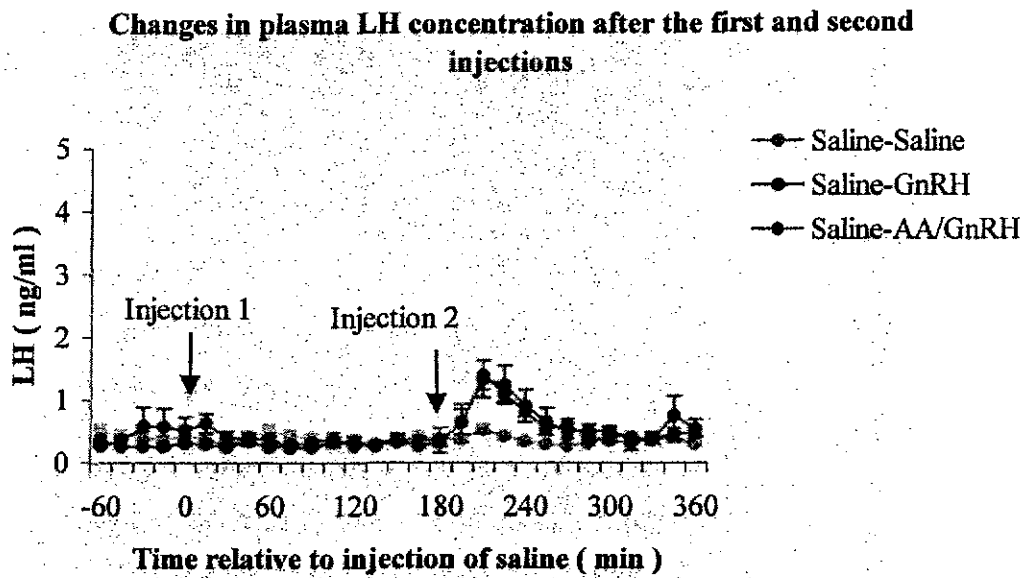


Figure 6.1. 3; Sheep did not respond to saline. Injection of GnRH caused a significant increase in LH secretion. Injection of amino acids together with GnRH caused no further increase.

Peak LH values for AA-GnRH and AA-AA/GnRH were 3.5 ± 0.99 and 4.16 ± 0.73 respectively.

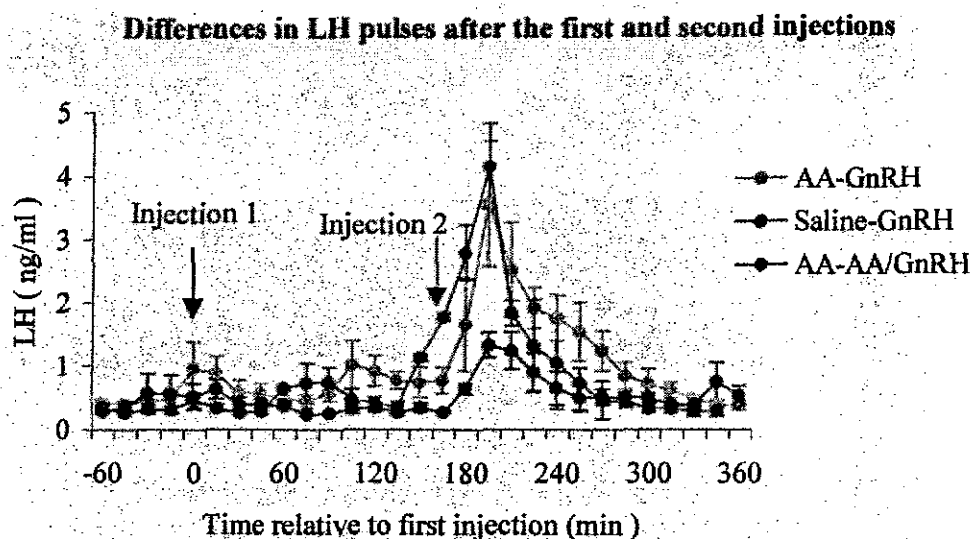


Figure 6.1. 4; Amino acid injected first (at 0 time) significantly increased LH secretion to GnRH at the second injection. There was a non-significant further increase in LH secretion when the amino acid and GnRH injected together.

EXPERIMENT 2

To confirm if Serine/Threonine injection can increase basal LH secretion, 7 sheep were injected with saline and 8 sheep injected with amino acids 5 times every 2h intervals starting at 10:00 (Figure 6.1.5). The injection of amino acid did not increase basal LH secretion.

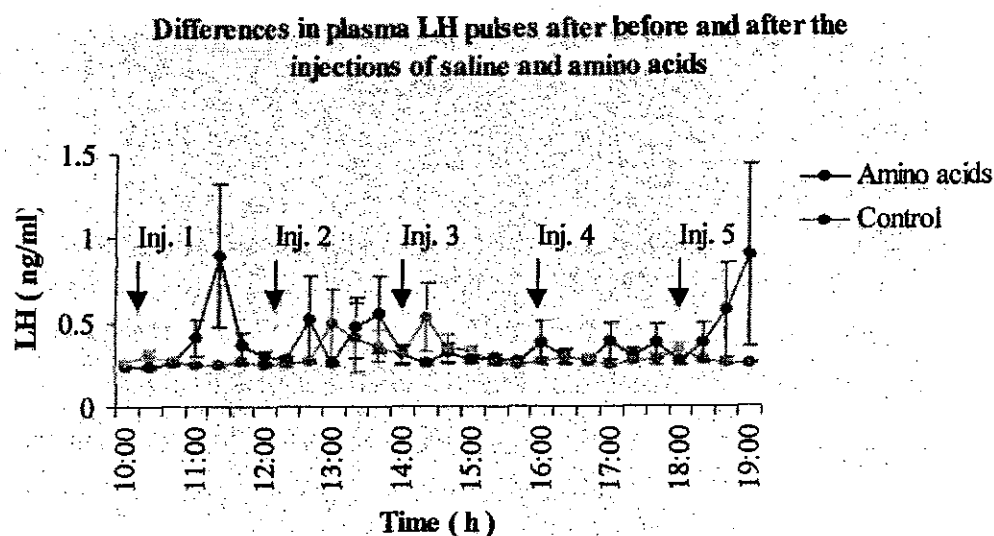


Figure 6.1.5; Seven sheep injected with saline (control) and eight sheep were injected with 2gr L-Serine and 1g L-Threonine dissolved in 20ml saline (Amino acids). Blood samples were taken for 9 hours at 20 min intervals. The difference between the two group is not significant ($P>0.05$).

EXPERIMENT 3

The objective of this work was to test if Azagly-naferelin was a potent GnRH agonist. Therefore, the effect of different doses of GnRH and GnRH analogue on LH secretion were compared. Low doses of analogue were not effective at inducing LH pulses, whereas higher doses were very effective (Figure 6.1.6). Higher doses of the analogue injection caused significant increases in LH pulses. Peak LH pulse concentration for saline, 0.1ng and 1ng analogue are 1.18 ± 0.38 , 6.60 ± 1.80 and 46.21 ± 6.82 (Mean $\pm$ sem) respectively (Figure 6.1.7).

Changes in LH secretion after the injections of saline and GnRH analogue

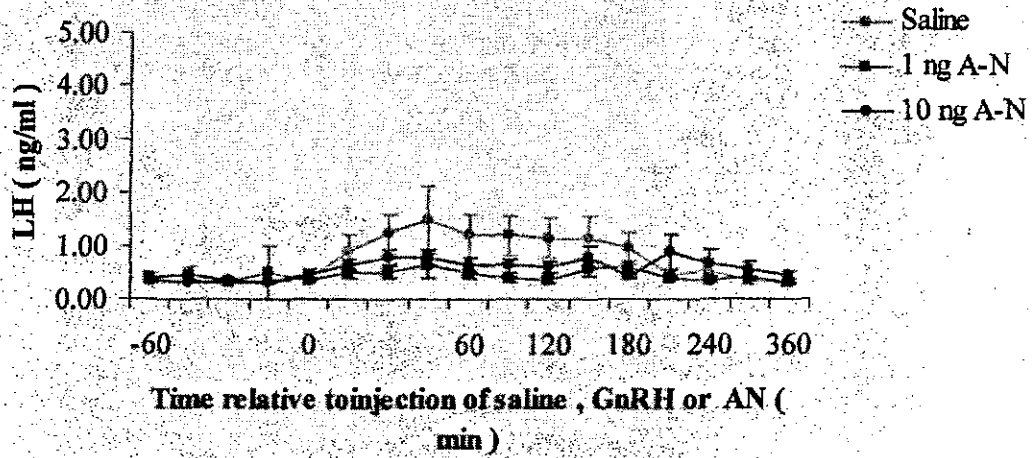


Figure 6.1.6; Small doses (1ng A-N) of GnRH analogue did not cause any change in LH secretion. When the dose was 10 times high (10ng A-N), LH secretion was increased, but it is still below saline. Ewes showed some response to saline. Differences are not significant ($p > 0.05$)

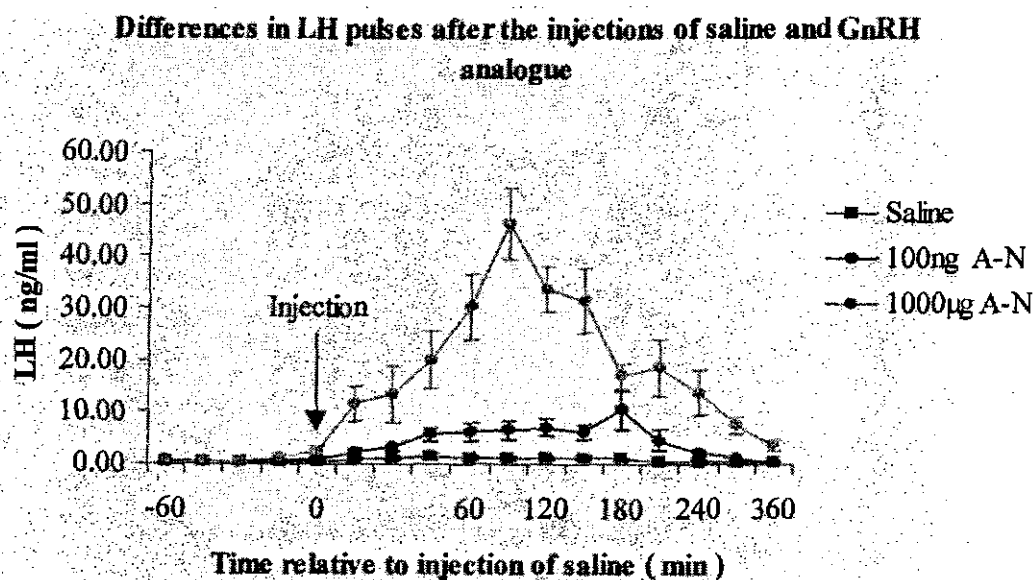


Figure 6.1.7; Injection of 100 and 1000ng GnRH analogue caused large increases in LH secretion ($p < 0.05$) (Note difference in scale to Figure 6.1.6).

The injection of 1 and 10µg doses of GnRH caused significant increases ($p < 0.05$) in LH secretion (Figure 6.1.8). Peak LH level for 0.1, 1 and 10µg GnRH were 2.04 ± 0.48 , 5.01 ± 0.83 and 12.55 ± 2.66 respectively.

Differences in LH secretion in response to increased doses of GnRH

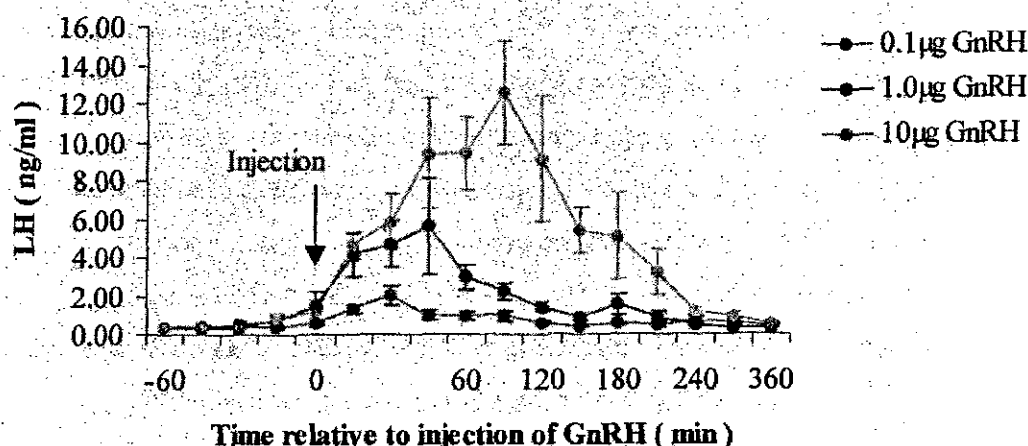


Figure 6.1.8; LH secretion was significantly increased after the injections of 1 and 10ng doses of GnRH.

DISCUSSION

The response of sheep to the injection of amino acids and GnRH was diverse. The response was large in some animal, whereas it was small in others. Again, some animals showed immediate responses to the injections, whereas some of the others responded later. According to Clarke, (1995), this late increase in LH, in some animals, is because the secretion of LH from the pituitary gland is inhibited very soon before the onset of LH surge by inhibitory factors. This factor could be oestrogen, or some other pituitary feedback mechanism. LH and FSH secretion are differentially regulated by GnRH pulses. The mechanisms involved, in regulation of gonadotrophin response of pituitary to GnRH, are not understood. These mechanisms might include the regulation of GnRH receptors, involvement of intermediates (Activin and follistatin) and second messengers (Ca^{2+}). Immunisation of sheep, with an ovine anti-GnRH antiserum, depressed pulsatile LH secretion and decreased plasma FSH levels. Pulsatile administration of the GnRH analogue (desGly10 ethylamide) to immunised sheep restored pulsatility of LH (Molter-Gerard *et al.*, 1999). The results of experiment 1, presented here, shows that amino acids injected 4h before GnRH

injection significantly stimulated LH secretion. The injection of amino acids together with GnRH caused a non-significant further increase in LH secretion.

Injection of GnRH caused significant increase in LH secretion. Lower doses of GnRH or GnRH analogues were not effective in inducing LH pulses, whereas higher doses of analogue cause a large increase in LH secretion. Injection of the rams with increasing doses of GnRH elicited a dose-dependent rise in LH secretion (Wu *et al.*, 1987). A similar result was obtained from female sheep, which were injected with 125, 250 ng GnRH in saline. Mean plasma concentrations of LH were significantly higher in animals receiving 250ng GnRH than those receiving 125ng GnRH or saline (McLeod and Haresign, 1984). Clarke, (1995) reported that injection of sheep with 500 ng GnRH in 4 pulses of 125ng, at 10 min intervals, caused a small increase in LH secretion. This injection did not cause a preovulatory LH surge. Injection of a bolus amount of GnRH (1000 ng) induced a preovulatory LH surge.

The results of experiment 3 show that the analogue is about 38 times more potent than natural GnRH in stimulating LH secretion. In experiment 1, we injected 150 ng GnRH which is a smaller dose than that injected by Clarke, (1995). This dose caused a small increase in LH secretion. The effect of specific nutrients (such as branched chain and excitatory amino acids) on gonadotrophin secretion was investigated by Downing *et al.*, (1995) and Honaramooz *et al.*, (1998). They injected sheep with branched chain amino acids and reported that injection has no effect on LH and FSH secretion.

The results of experiment 1, in this chapter, show that injection of amino acid increased LH secretion significantly. In the second experiment, amino acid injection did not effect basal LH secretion. This may be because some animals did not respond whereas others did. The reason for this is not known and it is difficult to predict the sheep, that will respond to an injection of amino acids. The reason for the positive effect of amino acid injection on LH secretion may be a result of an ovarian effect on the pituitary response of gonadotrophs to GnRH. As proposed earlier, Serine/Threonine amino acids are substituted by intracellular proteins and phosphorylated. This phosphorylation is a key factor for the production of ovarian steroids, activation of intracellular proteins involved in gene transcription (Such as CREM and CREB proteins) and the production of TGF β like peptides (Potchinsky *et*

al., 1997) which may have direct or indirect feedback effect on secretion of gonadotrophins.

The results presented here shows that Serine and Threonine amino acids increase the secretion of LH (experiment 1, figure 6.1.4) possibly by increasing activity of GnRH secretory cells in hypothalamus or by increasing the sensitivity of pituitary gonadotrophs to GnRH probably caused by oestradiol. This could be confirmed by *in vitro* work using pituitary cell culture to measure the response of pituitary cells to different concentrations of Serine and Threonine in the presence of GnRH.

CHAPTER VII; THE EFFECT OF AMINO ACID ON FSH SECRETION

7.1. THE ACUTE EFFECT OF INJECTING A MIXTURE OF SERINE AND THREONINE ON PLASMA CONCENTRATIONS OF FSH IN ANOESTROUS EWES TREATED WITH GnRH OR A GnRH AGONIST, AZAGLY-NAFARELINE.

Abstract

This experiment was designed to test the effect of injecting a mixture of Serine and Threonine on FSH secretion. Sixteen naturally cycling ewes were injected with a mixture of Serine/Threonine and GnRH or a GnRH agonist to test their effects on plasma concentrations of FSH. Injection of the amino acids tended to suppress the concentration of FSH. Mean concentrations of FSH tended to be further reduced when the amino acids were injected at the same time as GnRH. Injection of GnRH caused little increase in FSH secretion. The mean concentration of plasma FSH for Saline-Saline; Saline-GnRH; Serine/Threonine-Serine/Threonine plus GnRH; Serine/Threonine-GnRH and Saline-Serine/Threonine plus GnRH were 1.06 ± 0.16 ; 1.26 ± 0.22 ; 0.99 ± 0.16 ; 1.21 ± 0.36 and 0.83 ± 0.10 respectively. This experiment showed that the injection of amino acids tended to reduce plasma concentrations of FSH and that this reduction was greater when the amino acids were injected at the same time as GnRH. The reduction in FSH secretion is probably the result of a decrease in the pituitary response to GnRH or it may be the result of an increased negative feedback of ovarian factors on FSH secretion. These factors might be steroid or TGF- β like factors. Because, intra-venous injection of bovine follicular fluid, which is rich in inhibin, suppressed plasma concentration of FSH whereas the frequency and amplitude of LH pulses was increased.

INTRODUCTION

Pituitary gonadotrophins play an important role in ovarian follicular development and steroidogenesis. Nutrition can alter gonadotrophin secretion via an effect on pituitary sensitivity to GnRH, by a direct effect on GnRH neurones or by the feedback effects of hormones produced by the ovary. In ovariectomised ewes with a high food intake, the sensitivity of the pituitary has been reported to be higher than that of the ewes fed a moderate food intake (Rhind *et al.*, 1989b). It has been reported that specific components of the diets (fatty acid and glucose and amino acids) are important for the increase in ovulation rate (Blache *et al.*, 1996; Wade *et al.*, 1996). Fatty acid and glucose are oxidised for energy within the tissues. Energy availability important for the activity of GnRH secreting neurons. Inhibition of fatty acid and glucose oxidation by pharmacological substances has been reported to inhibit GnRH and gonadotrophin secretion (Wade *et al.*, 1993). Therefore, specific components of food affect reproductive status of animal.

Some amino acids are important in reproduction because they are taken up by intracellular proteins and phosphorylated which is important for steroidogenesis. Serine and Threonine amino acids can affect GnRH secretion due to an ovarian steroidal feedback effect influencing gonadotrophin secretion which is important for ovarian function. Follicular responses to LH pulses of different amplitude depend on both the stage of development of the follicle and the peripheral concentrations of FSH. Short-term treatment with a GnRH agonist does not generally suppress plasma concentration of FSH whereas chronic treatment with a GnRH agonist will eventually suppress plasma concentrations of FSH. In the hypogonadal ewe, in the absence of pulsatile LH secretion, FSH alone will stimulate the growth and development of large oestrogenic follicles which are competent to ovulate in response to a suitable exogenous stimulus of LH (Picton and McNeilly, 1990; Picton *et al.*, 1990).

In this experiment, sheep were injected with a mixture of Serine and Threonine to test the effect of this combination of amino acids on FSH secretion. If these amino acids have an effect on hypothalamo-pituitary axis due to the oestradiol production and expression of TGF- β like factors effecting the secretion of FSH. These amino acids may indirectly, regulate ovarian function through the expression of TGF- β like proteins

as result of phosphorylation of Serine and/or Threonine in intracellular proteins involved in signal transduction and gene transcription. Nuclear transcription factors, with a role in transcription of genes, such as cAMP-responsive enhancer elements binding protein (CREB) and cAMP-responsive enhancer element modulator (CREM) are generally phosphorylated on Serine and Threonine amino acids. The phosphorylation of CREB on serine 133 is important for gene transcription. CREB and CREM proteins regulate expression of mRNA for TGF- β like peptides (Potchinsky *et al.*, 1997).

MATERIAL AND METHODS

The plasma samples collected for LH assay were analysed for FSH.

Iodination

FSH for iodination (Cat; AFP 4117A) was obtained from NIDDK-NIH, Beltsville, and USA. 5 μ g of FSH was iodinated using the chloramine-T method as described chapter 6. The result of an iodination displayed in figure 7.1. 1.

Reference preparation

oFSH (APB-4117A) obtained from USA-BARC-Reproduction Lab-Beltsville, USA. Standards were prepared in assay buffer in concentrations of 0.05 to 50 ng/ml (Figure 7.1.1).

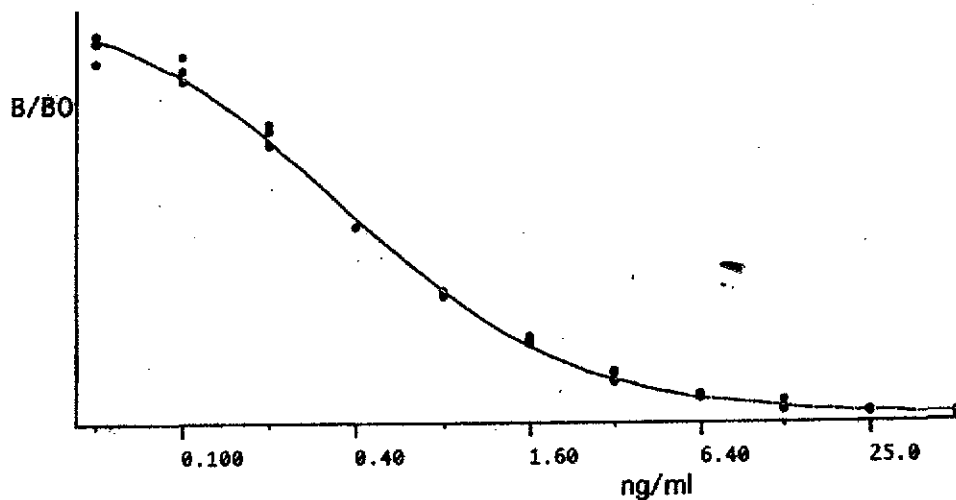


Figure 7.1.1; Standards for FSH made up from 0.05 ng/ml to 50ng/ml.

FSH Assay

Anti-ovine FSH developed in the rabbit (Cat; APF-C5288113, NIDDK-NIH, Beltsville, USA) was used at a dilution of 1/12000. Anti-rabbit IgG (second antibody) and normal rabbit serum were the same as used for LH assay (chapter 4). Buffers, incubation time and other assay procedures applied in FSH assay were also identical to the LH assay.

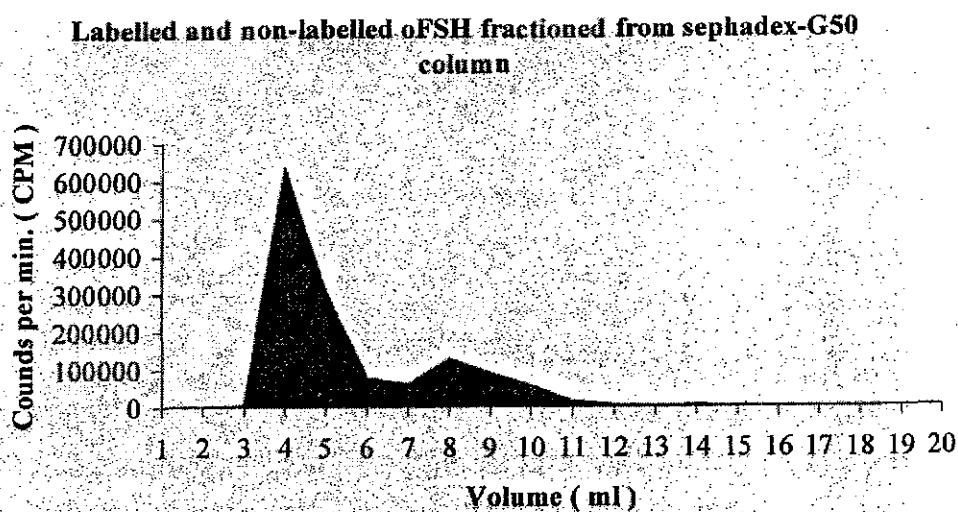


Figure 7.1.2; oFSH iodinated by using the Chloramine-T method. Approximately 1ml fractions (n= 20) were collected and 2µl of each fraction were counted in a gamma counter. Fraction 4 and 5 used as label for the FSH assay.

Assay parameters

Assay parameters were calculated in same way as used for LH assay. The sensitivity of FSH assay was 0.055 ± 0.005 ng/ml. Inter-assay coefficient of variations for high, medium and low were 7.4, 7.6 and 12.0. Intra-assay coefficient of variations were 4.8, 16.9, 17.9 respectively.

Statistical analysis

Data were analysed using randomised block design same as used for LH assay (Chapter IV).

RESULTS

EXPERIMENT 1

FSH secretion increased slightly (not significantly) after the second injection of saline. Injection of 150ng/ml GnRH caused a significant increase in FSH secretion (Figure 7.1.3). The injection of amino acids at the same time as GnRH suppressed FSH secretion significantly ($P<0.05$).

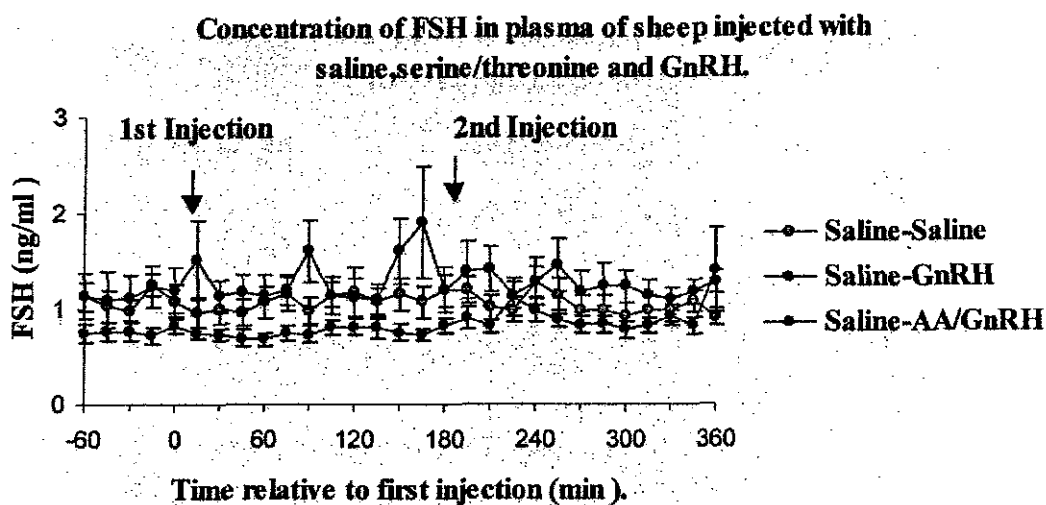


Figure 7.1.3; Sheep were injected twice (injection 1 and injection 2) with Saline-Saline, Saline-GnRH, Saline- AA/GnRH. Increase in FSH secretion was significantly higher in saline-GnRH group as compared with saline-saline and saline-AA/GnRH groups. The secretion of FSH was significantly reduced in saline-AA/GnRH group.

When sheep were injected first with amino acids and second with GnRH, plasma FSH concentrations tended to reduce and this reduction was significant when the first injection was the amino acids and the second was amino acids plus GnRH as compared with saline- GnRH group (Figure 7.1.4).

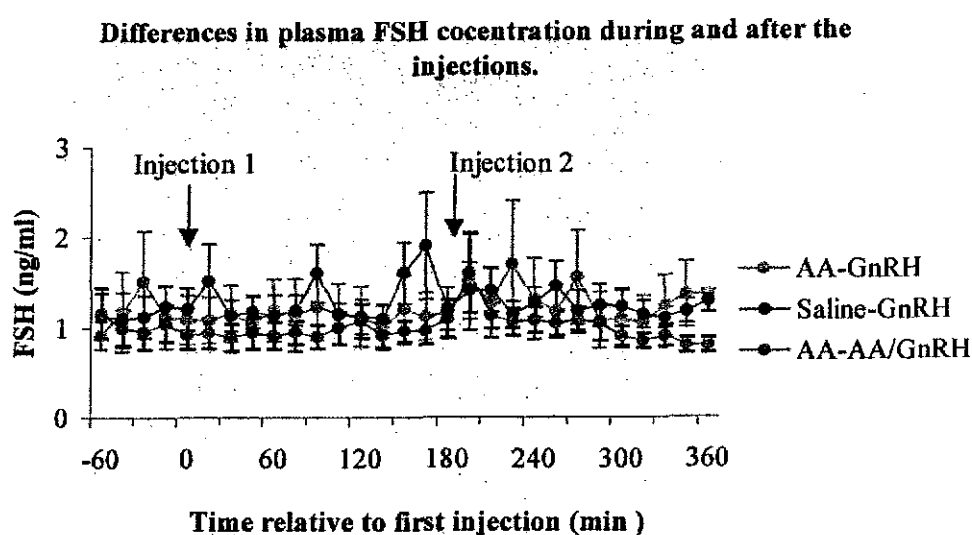


Figure 7.1.4; The injection of the amino acids and then the amino acid together with GnRH caused a significant reduction in FSH secretion.

EXPERIMENT 2

Sheep were divided into two groups and injected with saline (Control) and amino acids (Test) every two hours from 10 am to 6 pm. The injection of the amino acids significantly suppressed FSH secretion ($F=187.6$; $P_{0.05-0.01}=4.21-7.68$). After the 4th injection, the differences between saline and amino acid injected groups were greater than the differences seen after 1st, 2nd, and 3rd injections (Figure 7.1.5.).

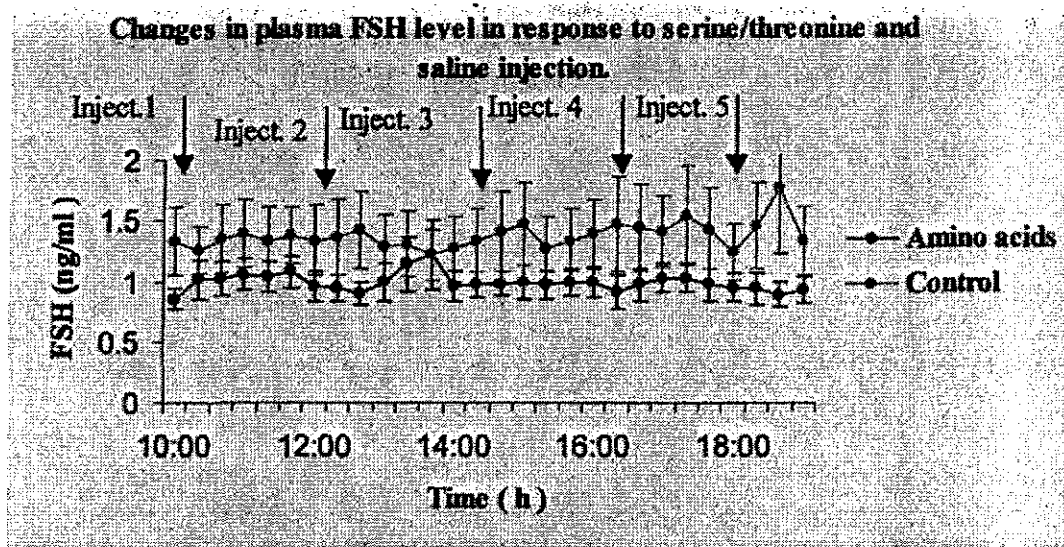


Figure 7.1.5; Eight sheep were injected 5 times with Serine/Threonine and seven sheep were injected 5 times with saline. The differences were not significant between injection1 and 3, but after the 4th injection, the differences became significant ($P<0.05$).

EXPERIMENT 3

The injection of sheep with small doses 0.1 and 1 μ g GnRH did not cause any significant change in FSH secretion (Figure 7.1.8). The injection of a higher dose (1 μ g) increased FSH secretion but not significantly (10 μ g) caused a significant increase in FSH secretion ($p<0.05$).

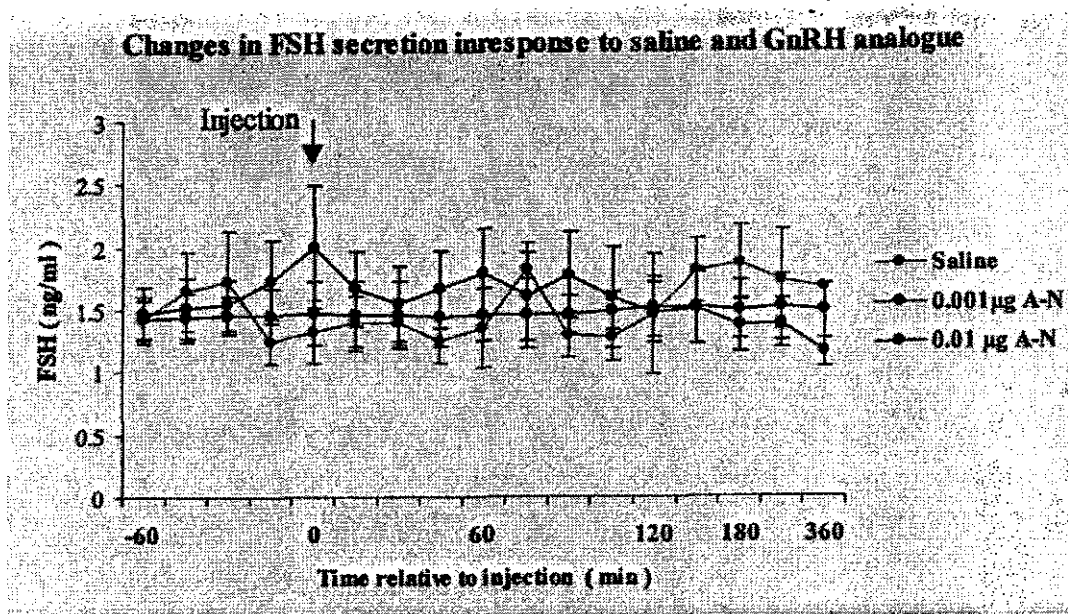


Figure 7.1.6; There was no effect of the GnRH analogue (azagli-naferelin) at the doses of 0.001 and 0.01 µg/ml, on FSH concentrations.

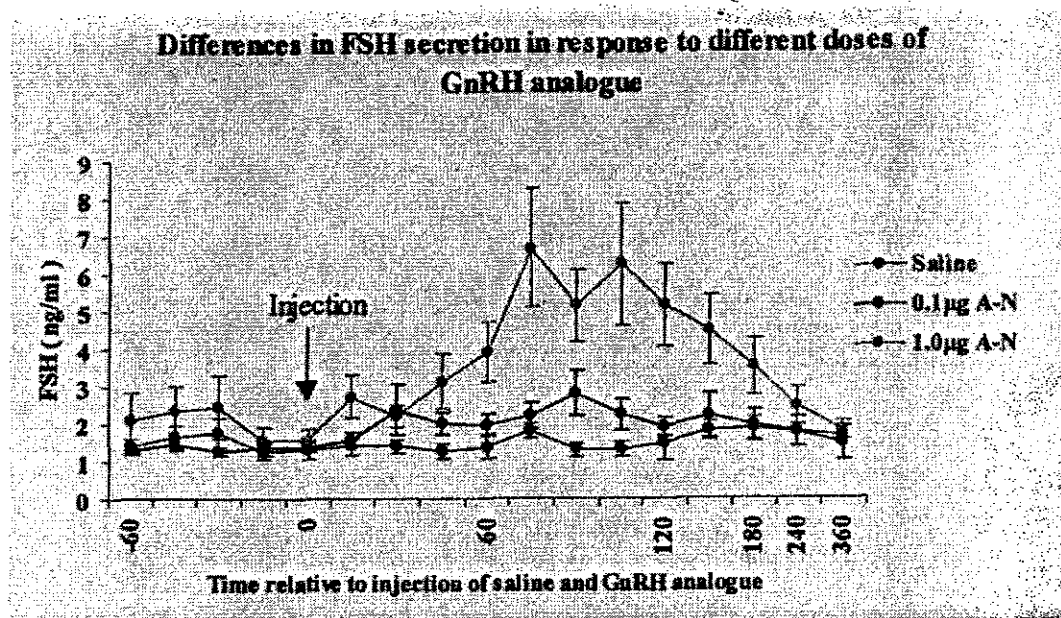


Figure 7.1.7; The injection of 1 ng of the GnRH analogue significantly increased plasma FSH concentrations ($P < 0.05$) while the injection of 0.1 µg A-N caused a smaller significant increase.

GnRH is not as potent as the analogue, which was 30 times more potent than GnRH in stimulating FSH secretion.

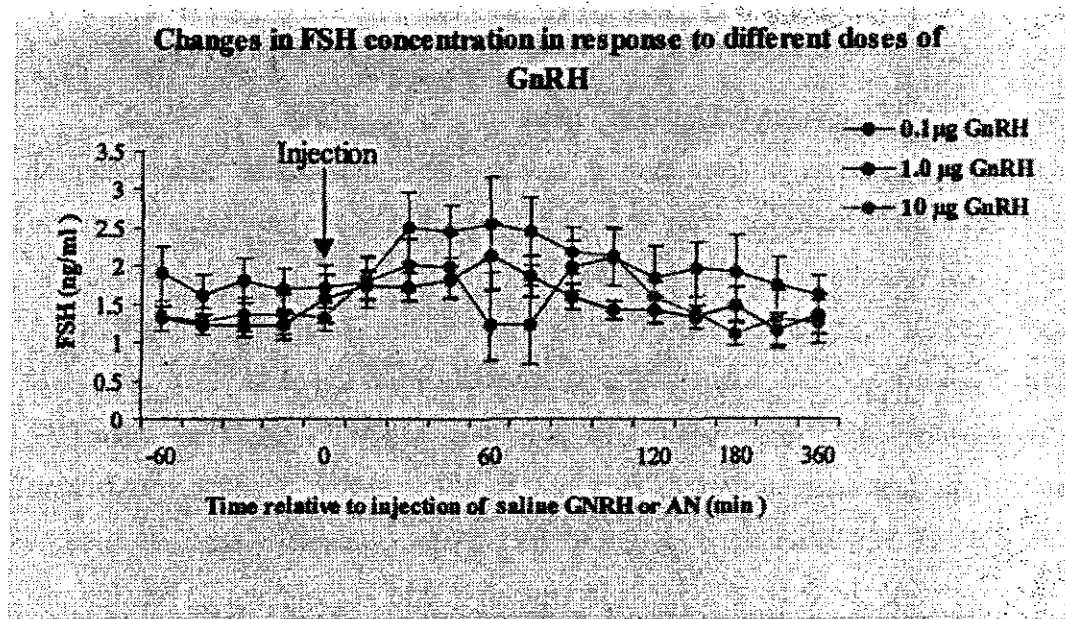


Figure 7.1.8; Low doses of GnRH were ineffective in increasing FSH secretion, at 10ng, GnRH stimulated FSH secretion.

DISCUSSION

Small doses of GnRH and the GnRH analogue were not effective in stimulating FSH secretion. However, higher doses of GnRH (10µg) and of Azagly-neferelin (1.0 µg) caused a significant increase in plasma FSH concentration. Azagly-neferelin was 30 times more potent than GnRH in stimulating FSH secretion.

The injection of amino acids suppressed the plasma concentration of FSH whereas there was no difference in the secretion of LH (chapter 6). This effect of amino acid injection could be a result of an increased negative feedback effect of ovarian factors. These ovarian factors could be inhibin and oestradiol. Ovarian secretion of oestradiol is under the acute control of LH. In chapter 6, secretion of LH increased by the injection of a mixture of Serine and Threonine. These were the same animals injected with the same amino acids (chapter 7) and the secretion of FSH was reduced. It is possible that, the increase in LH secretion caused an increase in oestradiol secretion, which suppressed FSH by negative feedback.

Increase in oestradiol secretion because of the increase in LH is not clearly known but it is possible that the increase in LH secretion increased androgen substrate for aromatisation or by a direct action *via* the accumulation of LH-receptor expression on granulosa cells (Baird and McNeilly, 1981; Webb *et al.*, 1981; Campbell *et al.*, 1990; Evans *et al.*, 1996).

In this experiment, the factor causing FSH suppression might be inhibin rather than oestradiol. Because inhibin exerts acute control over the secretion of FSH. The effects of inhibin, on FSH secretion in sheep can be partly mediated at the level of the pituitary GnRH receptor, but in the rat it has been suggested that inhibin reduced FSH secretion by specifically blocking synthesis of the hormone. It has been suggested that low doses of inhibin do not cause any change in LH secretion, but low doses still effect FSH secretion. Martin (1986), injected sheep with charcoal-extracted bovine follicular fluid (bFF), which is rich in inhibin, throughout the luteal phase and significantly reduced plasma concentrations of FSH and increased secretion of LH. LH secretion was effected by the injection of bFF because high doses (9 ml, 3.65ku/ml) were used and high doses of inhibin increased LH secretion.

Inhibin is a member of the TGF β family. It can be said that TGF- β -like proteins block FSH secretion. Serine/Threonine injection may increase expression of TGF β like proteins effecting the secretion of FSH. The expression of TGF- β -like proteins might be increased by Serine/Threonine phosphorylation because this phosphorylation leads to production of TGF- β -like proteins that could then exert a negative effect on secretion of FSH.

Serine and Threonine amino acids may have also had an effect at the level of the hypothalamus or pituitary, reducing FSH secretion. Serine and Threonine may have caused a reduction in the sensitivity of FSH secretory cell to GnRH or a decrease in FSH releasing hormone secretion from the hypothalamus.

CHAPTER VIII; GENE EXPRESSION

8.1 THE EFFECT OF INJECTING A MIXTURE OF SERINE AND THREONINE ON THE EXPRESSION OF mRNA'S FOR TGF- $\beta_{1,2,3}$, FSHR, LHR, P450_{SSC} AND P450_{AROM} WITHIN THE MOUSE TESTIS AND THE PMSG PRIMED MOUSE OVARY.

Abstract

The aim of this experiment was to test the effect of injecting a mixture of serine and threonine on the expression of mRNA's for TGF- $\beta_{1,2,3}$, FSHR, LHR, P450_{SSC} and P450_{arom} within the mouse testis and ovary. Male (n=60) and Female mice (CD-1 strain; n=60) were injected with saline (Control), a mixture of Serine and Threonine (A) or Glycine (G). On the third day of treatment, the female mice were injected with 2.5 iu PMSG. Total RNA was extracted, transferred onto a nylon membrane and probed with ³²P-labelled cDNA probes. The expression of mRNA's for TGF- β_1 and β_3 was increased by Serine/Threonine and Glycine injection in both male and female mice. The expression of mRNA for TGF- β_2 was increased by Serine/Threonine injection in the male but there was no difference in the female. Expression of mRNA for the gonadotrophin receptors and the steroidogenic enzymes was increased in the testis, but decreased in the ovary following Serine/Threonine injection. These data show that the three isoforms of TGF- β are expressed in the ovary and testis and that their expression can be increased by the injection of a mixture of Serine and Threonine.

INTRODUCTION

It was hypothesised that; nutrition may effect reproduction by means of certain nutrients in the diet such as specific amino acids. The injection of a mixture of serine and threonine may alter the expression of mRNA's for Transforming Growth Factor- β (TGF- β) and therefore alter the expression of mRNA's for the gonadotrophin receptors, which in turn, may change the expression of mRNA's for enzymes involved in steroid production in the testis and ovary.

There are several species of TGF- β ; TGF- β_1 , β_2 , β_3 , β_4 and β_5 . TGF- β_1 , β_2 and β_3 all have similar activities depending on cell type. TGF- β_1 was purified first, from platelets and additional TGF- β like molecules have been since identified, all have 64-85% homology with each other. TGF- β_2 was initially identified by its ability to inhibit cell growth. TGF- $\beta_{3,4}$ and β_5 have been identified by cross hybridisation of cDNA probes under reduced stringency (O'Reilly *et al.*, 1992).

Transforming Growth Factor- β like molecules modulate several biological activities of theca and granulosa cells, including growth, differentiation and the expression of mRNA's for gonadotrophin receptors and steroidogenic enzymes (eg. P450 arom. and P450scc.) (Junta *et al.*, 1996). The granulosa and theca cells of small developing preovulatory follicles express mRNA for several growth factors (Adashi, 1991; Chegini and Williams, 1992; Vanderstichele *et al.*, 1994). The expression of mRNA for TGF- β_1 , β_2 and β_3 are differentially regulated, for example oestrogen decreases mRNA expression for TGF- β_1 and TGF- β_2 but, not TGF- β_3 (Adashi, 1991; Vanderstichele *et al.*, 1994)

Ovarian androgen production is regulated by TGF- β_1 (Hernandez *et al.*, 1990). Transforming Growth Factor- β_1 acts directly on interfollicular tissue to inhibit androgen production while increasing progesterone secretion. These functions can be mimicked by other members of TGF- β /inhibin family (Magoffin *et al.*, 1989). TGF- β_1 also acts on granulosa cell to increase progesterone production in response to FSH. In the ovary, TGF- β_1 stimulates FSH induced inhibin production and LH induced progesterone secretion (Juneja *et al.*, 1996).

TGF β_1 modulates FSH and LH receptor expression within the ovary. It has both stimulatory and inhibitory effects on LHR expression. Small doses (5 ng/ml) of TGF- β_1 stimulates LHR expression whereas higher doses (50 ng/ml) are inhibitory (Knecht *et al.*, 1987). FSH and LH receptors are the members of a family of receptors that act through G proteins and the genes for these receptors have been characterised (Heckert and Griswold 1993; Dufau *et al.*, 1995). The genes coding for gonadotrophin receptors have a number of similarities. First they are very large with 10 or 11 exons and second, the last exon in each gene codes the entire transmembrane and intracellular

region and third, exons 2 and 9 code for external domain of the receptors (Heckert and Griswold, 1993).

In the testis, Leydig cell and Sertoli cell produce TGF- β s in a latent form and their production is decreased by gonadotrophins. Follicle Stimulating Hormone has been reported to decrease TGF- β secretion from immature porcine Sertoli cells (Muhleron and Schomberg 1990). Similarly, its production from Leydig cell was decreased by LH (Avallet *et al.*, 1991).

Leydig cells produce testosterone regulating testicular development and maintenance of spermatogenesis. Androgen is converted to oestrogen by P450_{arom} in the Sertoli cells within the testis. Recently it has been reported that, TGF- β s reduce the number of gonocytes by decreasing their proliferation or reducing their survival. TGF- β acts on Leydig cells or gonocytes rather than Sertoli cells because Sertoli cells do not have receptors for TGF- β s (Olaso *et al.*, 1998).

Leydig cells provide androgen to seminiferous tubules to maintain spermatogenic function (Lejeune *et al.*, 1998). Decreased spermatid number could result from the reduced production of testosterone associated with the production of TGF- β like peptides within the testis, since TGF- β modulates gonadotrophin regulated androgen production.

Therefore, TGF- β 's can modulate steroidogenic functions of the testis and ovary effecting gametogenesis. It was hypothesised that, the injection of a mixture of Serine and Threonine could alter the expression of TGF- β like proteins in the testis and ovary hence affecting hormonal balance and the modulation of gametogenesis.

METERIAL AND METHODS

Experimental design

Male and female mice (n=60) were divided into six groups and injected for 5 days with saline (control), serine and threonine (test) and glycine (positive control) as shown on the table 8.1.1.

Table 8.1.1; Experimental plan

Groups	Sex		Injections				
	Male	Female	Day 1	Day 2	Day 3	Day 4	Day 5
Number of animals	30	30					
Saline	10	10	S	S	S + 2.5iu PMSG*	S	S, kill and collect gonads 1h later
Serine/ Threonine	10	10	A	A	A + PMSG	A	A, kill and collect gonads 1h later
Glycine	10	10	G	G	G + PMSG	G	G, kill and collect gonads 1h later

*Only female mice were given PMSG. S= saline, A= Serine and Threonine, G=Glycine (positive control)

a) Animals and injections

Nineteen and twenty one day old male (n=30) and female (n=30) mice (CD-1 strain) were injected daily for 5 days with saline, as a control or a mixture of Serine/Threonine or Glycine. The Serine/Threonine prepared as described (chapter 5). In this experiment, male and female mice were injected daily with 38 ng Glycine (Cat; G-8898, Sigma, Steinheim, Germany) in 0.2 ml saline for 5 days. On the third day of experiment, the females were injected intra-peritonealy with 2.5 iu PMSG (Cat; PL1708/4309, Intervet, Cambridge, UK) prepared as described (chapter 4). One hour after the last injection the mice were killed as previously described (chapter 4 and 5).

The ovaries and testis were removed under RNAase free conditions and immediately stored in liquid nitrogen until the extraction of total RNA.

b) Tissue preparation and isolation of total RNA

Stored tissue was ground in liquid nitrogen using a RNAase-free pestle and mortar. Ground tissue (100mg) was placed in 1ml Trizol reagent (Cat; 15596-026, 100 ml, Life Technologies. Paisley, Scotland), in 2ml micro-centrifuge tube (Cat; 02-1420-2700, Fisher, Leicestershire, UK)

c) Separation

The tubes containing the ground tissue and Trizol were left at room at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complexes. Then 0.2 ml of chloroform (0.2ml/1ml Trizol used) was added and the tube and shaken vigorously for 15 seconds then centrifuged at 1200g for 15 min at 4°C. The top colourless phase was transferred to a fresh eppendorf. The contents of each tube were mixed with 0.5 ml, per ml of Trizol, isopropyl alcohol (Cat; 405-7, Sigma. St. Louis, MO, USA), incubated at room temperature for 10 min. and centrifuged at 12000 g at 4°C for 10 min. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol (Cat; 200-578-x, Hyman Limited. Essex, England) and RNA recovered by centrifuging again at 7500g for 5 min at 4°C. Finally, the supernatant was removed, the RNA pellet dried briefly and then redissolved in 50µl nuclease free water which was prepared by adding 1ml diethyl pyrocarbonate (DEPC; Cat; D-5758, Sigma, Steinheim, Germany) to 1L dH₂O.

d) Quantification of total RNA

Total RNA was quantified by using a RNA/DNA spectrophotometer (Mod; 80-2103-98, Ser; 66884, Pharmacia Biotech. Cambridge, England).

e) REVERSE TRANSCRIPTION (RT)-POLIMERASE CHAIN REACTION (PCR)

For Reverse Transcription-Polimerase Chain Reaction (RT-PCR), two tube and two step procedure was applied (Figure 8.1.1.). In first tube, cDNA was synthesised and then an aliquot of that cDNA was transferred to a second tube for PCR.

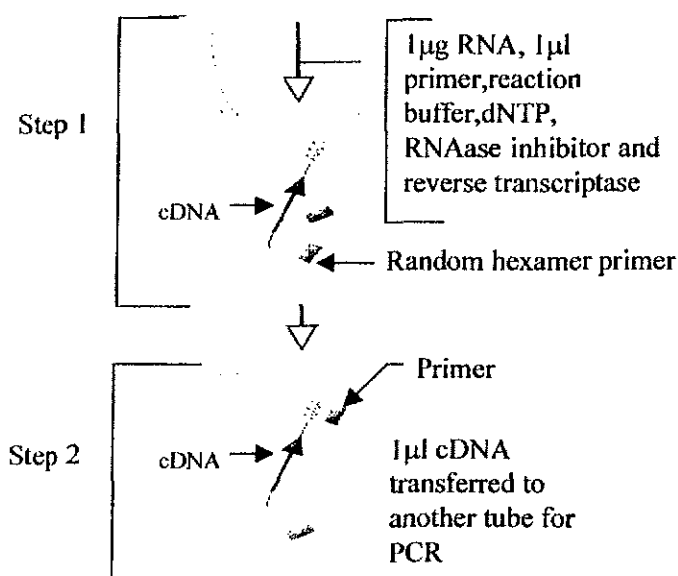


Figure 8.1.1. In step one, mRNA was transcribed to cDNA. In second step, an aliquot of cDNA transferred to another tube for PCR (Adopted from figure 36 of PCR application manual, Roche molecular biochemicals, second edition, p 96)

Reverse transcription(RT)

Reverse transcription (RT) is conversion of total RNA or mRNA to cDNA by reverse transcriptase. reverse transcription was done by using Advantage RT-PCR kit (Cat; K1402-1, Colontech, Palo Alto, USA). One µg of total RNA was placed in DEPC-treated water (in a final volume of 12.5 µl). Then, 1 µl of a random hexamer primer, (Random hexamer primer binds to mRNA at variety of complementary sites and lead to short cDNA's) was added to the tube and heated at 70°C for 2 min, quenched on ice and then following were added.

<u>Components</u>	<u>Volume in mix (µl)</u>
5x Reaction buffer	4
dNTP mix (10mM each)	1
RNAase inhibitor	0.5
<u>Reverse transcriptase</u>	<u>1</u>
Total volume	6.5µl

The contents of tubes were mixed by gentle pipetting and then incubated at 42°C for 1h and finally heated to 94°C to stop cDNA synthesis and to destroy any DNAase activity. The tubes were centrifuged for one min at 12000g at 4°C. The cDNA was aliquoted and stored at -70°C until its use in PCR.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is the amplification of certain mRNA sequence (by using specific primers for that mRNA) from primed cDNA by DNA polymerase (Brown, 1995). Then the mixture heated to 94° for the detachment of newly synthesised strands and cooled down for more primers to hybridise the cDNA at their respective position. This denaturation-hybridization synthesis is repeated usually 25-30 times to synthesise million copies of desired part of the cDNA. PCR reactions were set up by combining the following reagents in the indicated quantities.

<u>Component</u>	<u>Volume per reaction (μl)</u>	<u>Concentration in mix</u>
cDNA	1	1μg/reaction
dNTP (2.5mM)	4	0.2mM
Primers	1	0.2μM
10XPCR buffer	5	1X
Mg ⁺² (25mM)	5	2mM
Ampli Taq	1	5units/reaction
<u>PCR grade water</u>	<u>33</u>	-
Total volume	50μl	

The deoxynucleotide (dNTP) set with 2.5mM concentration (dATP, dCTP, dGTP and TTP) was obtained from Amersham Life Science (Cat; US77101, Ohio, USA). The other products were ordered from Perkin Elmer and supplied with the Ampli-Taq gold (Cat; H0595, Perkin Elmer, Jersey, USA). All the components were mixed and the PCR amplification reaction carried out using the Perkin Elmer GenAmp PCR system 9700. Amplification reaction was started with an initial denaturing reaction (10 min, 94°C), denaturing reaction (1 min, 94°C) then 38 to 58 cycles of annealing reaction (1 min, 58°C), elongation reaction (2 min, 72°C) and final

elongation (10 min, 72°C). After the PCR, 10µl PCR product and DNA size markers was run in 1% agarose gel for 1.5h at 65V and visualised under UV illumination.

a) Primers

Synthesized cDNA was amplified by using specific primers for TGFβ₁, β₂, β₃, LHR, FSHR, P450_{acc} and P450_{arom}. Primers for TGFβ₁, β₂ and β₃ kindly provided by Dr G. Zaman, Royal Veterinary College, Molecular Biology Unit, Royal College St. London, and NW1 OTU). Primers for LHR, FSHR, P450_{acc} and P450_{arom} kindly provided by Prof. PJ O'Shaughnessy, University of Glasgow Veterinary School Bearsden Rd, Glasgow G61 1QH.

c) Gel electrophoresis

Samples were run in 1% ultra pure agarose (Cat; 15510-019, Life Technologies, Paisley, Scotland). One-gram agarose was placed in a 500ml-glass flask and 100ml TEA buffer was added then placed in an oven to dissolve the agarose and allowed to boil until the agarose was completely dissolved. Then, 5µl of a 10mg/ml solution of Ethidium bromide (Cat; E-7637, Sigma, St Louis, MO, USA) was added, mixed gently and cooled down and then poured into an electrophoresis tank. When the gel was solid, it was placed in 1XTris ethylene acetate (TEA) buffer made up to 50X by adding 252 g Tris base (Cat; T-1503, Sigma, St Louis, MO, USA), 57.1ml Glacial Acetic Acid (Cat; 100001 8Q, BDH, Poole, England) and 100 ml 0.5 M EDTA (Cat; E-4884, Sigma, St Louis, MO, USA) then autoclaved.

The DNA ladder, size marker, 100bp (Cat; H0595, Perkin Elmer, Germany) was made up by mixing 60µl ladder with 10 µl gel loading solution (Cat; G-2526, Sigma, Steinheim, Germany) from this 5µl was loaded. Both PCR product and DNA size marker were diluted in loading buffer and run at 65V for 1.5h and then photographed under UV illumination.

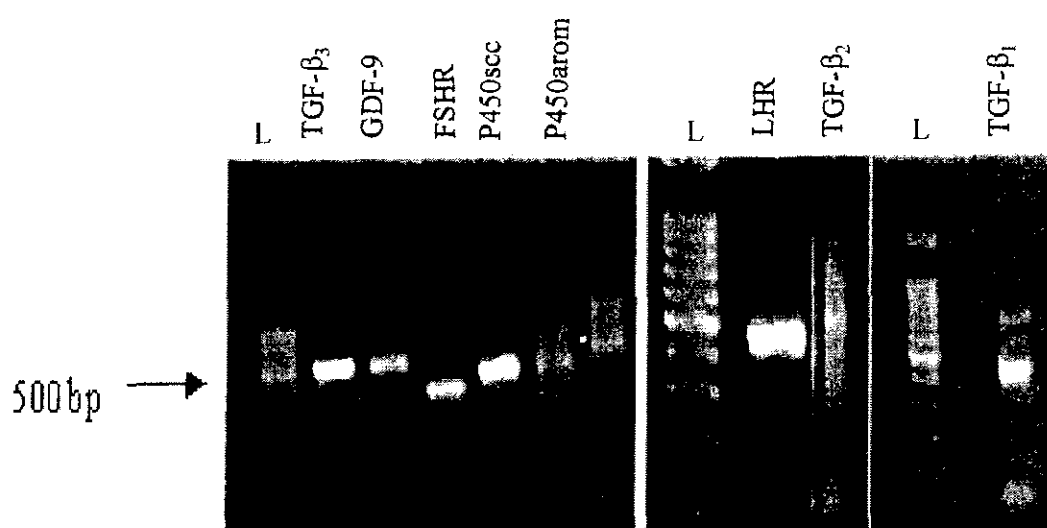


Figure 8.1.1; One μ l of primers added to each reaction tube. Primers for LHR, FSHR, P450arom and P450scc enzymes gave approximately 450bp, 350bp, 400bp and 400bp PCR product. Primers for TGF- β 1, β 2 and β 3 gave approximately 480, 593 and 524bp PCR product. The PCR products for TGF- β 2 and P450scc were removed by gel extraction. Extraction was performed by using a kit; Quick gel extractions kit (Cat; 28704, Qiagen, Hilden, Germany). 100bp DNA ladder (L) was used as a size marker

NORHERN BLOTHING

Buffers

a) 20X Standard saline citrate (20XSSC)

This buffer prepared by dissolving; 3M Sodium chloride (NaCl; Cat; 102415K, BDH, Poole, England), 0.3M Sodium acetate (CH₃.COONa; Cat; 44389, BDH, Poole, England) in 1L of diethyl pyrocarbonate (DEPC)-treated water and the pH was adjusted to 7 then it was autoclaved.

b) 0.5 M EDTA Solution

37.22g EDTA (Cat; E-4884, Sigma. St Louis, USA) added to 200-ml DEPC-treated water, the pH adjusted to 8 and the solution was autoclaved.

c) 10X(3-[N-morpholino]propanesulfonic acid (MOPS)

This buffer was used as an electrophoresis buffer 10X MOPS prepared by combining the followings.

0.2 M MOPS, C₇H₁₅NO₄S, (Cat; M-8899, Sigma. Steinheim, Germany)

50mM Sodium Citrate, C₆H₅Na₃O₇.2H₂O, (Cat; S-4641, Sigma, Steinheim, Germany).

10mM EDTA. The reagents were dissolved in 500-ml DEPC-treated water, pH adjusted to 7 and the buffer autoclaved.

d) Gel running buffer

This buffer was made up as 10XMOPS (w/w) and 5% (w/w) formaldehyde (Cat; F-8775, Sigma. St Louis, MO, USA).

e) Electrophoresis sample buffer

The buffer was freshly prepared by adding 0.75ml deionized formamide, 0.15 ml 10X MOPS, 0.24ml formaldehyde, 0.1ml glycerol and enough bromophenol blue (An electrophoresis reagent, sodium salt; Cat; B-8026, Sigma, St Louis, MO, USA) to produce a blue colour.

f) Making up the gel (1.2%)

Agarose (4.8g) was dissolved in 10X MOPS buffer by boiling until the agarose dissolved. In a fume cupboard, 20 ml formaldehyde was added to the gel at 60°C and mixed, It was then poured into an electrophoresis tank then the comp was placed on and left until the gel set. When the gel set solid, the gel-running buffer was added to cover the gel. This was prepared 2h before loading the samples.

g) Preparation of samples

Fifteen µg of total RNA from each experimental group was placed in 0.2 ml nuclease free micro tubes (Cat; AB-0337, Advanced Biotechnologies) and the volume made up to 15µl with sample buffer. The tubes, containing RNA plus sample buffer, were placed in a water bath at 61°C for 5 min and then immediately placed on ice, 1µl ethidium bromide (0.1mg/ml solution) was added. Fifteen µl was then loaded into the wells of the gel and run at 65V for 5h at room temperature (Figure 8.1.2a).

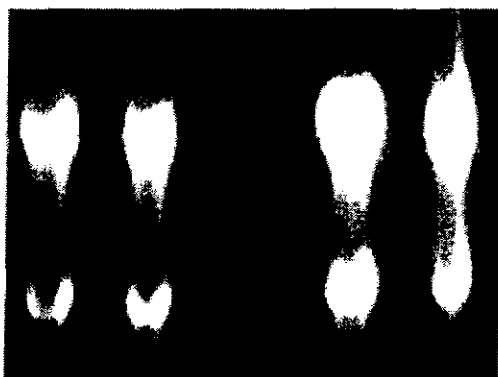


Figure 8.1.2a; 15 µg RNA run on gel containing 1.2% agarose in gel running buffer. The gel was run at 65V approximately 5 hours. During this time, ribosomal bands were spread.

h) Preparation of the gel for Northern transfer.

The gel was placed in saran film and photographed under UV illumination. The gel was then inverted and placed in a NaOH solution (2g NaOH in 1L DEPC water) for 40 min to produce sharper bands.

i) Setting up the transfer.

A plastic platform was placed in a 25X15cm plastic nuclease free container, Then a piece of 3mm Whatman filter paper was cut to the size of plastic platform and and immersed in transfer buffer (10X SSC). The wet filter paper was laid on the platform and pressed gently and slowly so that there were no bubbles between the platform and the filter paper. The paper covered the edges of platform, but did not touch the bottom of buffer container so as to facilitate upward transfer. Another piece of wet filter paper was placed on the top of platform without bubbles. The gel was taken out of the NaOH solution and placed on the filter paper that wells of the gel facing the platform. The edges of gel were protected from direct contact with the buffer. A piece of nucleic acid transfer membrane, Hybond-N+(Cat; I6435, Amersham, Buckinghamshire, England) cut to the size of the gel was placed on the gel. Another piece of wet filter paper was placed on membrane. To facilitate upward transfer of the buffer through the gel, by capillary movement, dry paper towels were stacked and a weight was placed on the gel and left approximately for 18 hours to transfer the RNA from the gel to the nylon membrane (Figure 8.1.2b).

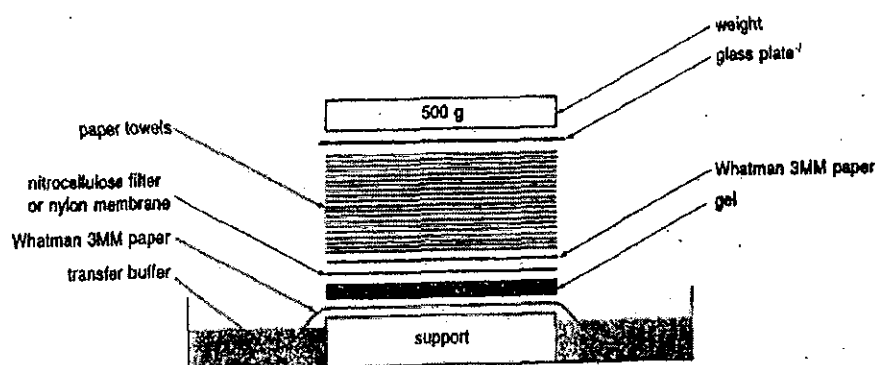


Figure 8.1.2b; RNA was transferred from agarose gel to nylon membrane by capillary action. The transfer buffer, from a reservoir, passes through the gel into a stack of paper towels. A weight placed on the paper towels to facilitate upward transfer.

j) Checking the transfer and preparation of the membrane.

When the paper towels were wet, they were removed and the RNA site on the membrane marked by pencil. The membrane was sandwiched between Whatman filter paper and left at room temperature for 10 min to dry. The gel was taken to a dark room to visualise the transfer. The membrane was placed in an oven at 80°C for 2h to fix the RNA. The dried membranes were kept in a secure place for hybridisation.

HYBRIDISATION

Hybridisation performed using a commercial kit (Multiprime DNA labelling systems; Cat; RPN, 1600Y, Amersham. Buckinghamshire, England).

a) Labelling of probes

Fifteen ng of a DNA probe (for labelling) was dissolved in 16.27µl of distilled and filtered water, denatured at 100°C for 5 min and then chilled on ice. The reaction was set up on ice as follows:

<u>Components</u>	<u>Volume in mix (µl)</u>
DNA solution	16.27
Unlabelled dNTP	2.4
Buffer	3
Primer (solution 2)	3
Water (To make a final volume of 30µl)	1.13
[α- ³² P] dATP (3000Ci/mmol)	3
<u>Enzyme (Klenow)</u>	<u>1.2</u>
Total volume	30µl

The tube was mixed gently by pipetting up and down and then centrifuged to concentrate the contents at the bottom of the tube. The tube placed in a water bath at 37°C, the water bath was then switched off and left overnight for labelling.

b) Prehybridisation

The membrane was removed from the filter cover and placed in a clear roller bottle, in de-ionised water (100 ml) to wash out the agarose and salts from the membrane before pre-hybridisation. The washing was done by rolling the bottle by hand for 1-2 min and then pouring the water down the sink. The membrane was then placed in 10 ml hybridisation buffer preheated to 68°C and incubated in hybridisation oven for 50 min.

c) Hybridisation

One ml hybridisation buffer was added to a screw cap tube, containing 30 µl labelled and denatured probe and an 100 µl of denatured salmon sperm DNA (Cat; D-1626, Sigma, Steinheim, Germany). The tube was then mixed and the contents was transferred to a bottle and left to hybridise for 1h.

d) Washing

One hour later the oven was opened and hybridisation buffer poured away. The blot was rinsed with 100 ml 2XSSC and 0.1X SDS (w/w) and washed with a mixture of 100ml 0.1XSSC and 0.1XSDS for 10 min at 37 °C and washed again with 100ml 0.1XSSC and 0.1XSDS at 60°C for 20 min. After washing, the blot was wrapped with PVC and monitored for radioactivity and then placed in a case with the RNA side next to the film. The blot was exposed at -70°C for 24h. After 24h, the case was opened under the safe red light and developed using a Konica SRX-101 film processor (X-Ograph imaging systems, Gloucestershire, England).

e) Quantification

Quantification was done by using molecular analyst software (Copyright 1992-1995, Bio-Rad Laboratories, Hercules, USA). For each RNA transcript, background optical density was dismissed and the pipetting errors were corrected by dividing each transcript by β -actin which was hybridised on the same membrane used for the hybridisation of TGF- β s, LH-R, PSH-R and P450 enzymes.

RESULTS

The injection of Serine /Threonine or Glycine (positive control) caused an increase in expression mRNA for TGF- β 1 within the mouse ovary. In the testis, Glycine injection caused a reduction in expression of mRNA for TGF- β 1 (Figure 8.1.3).

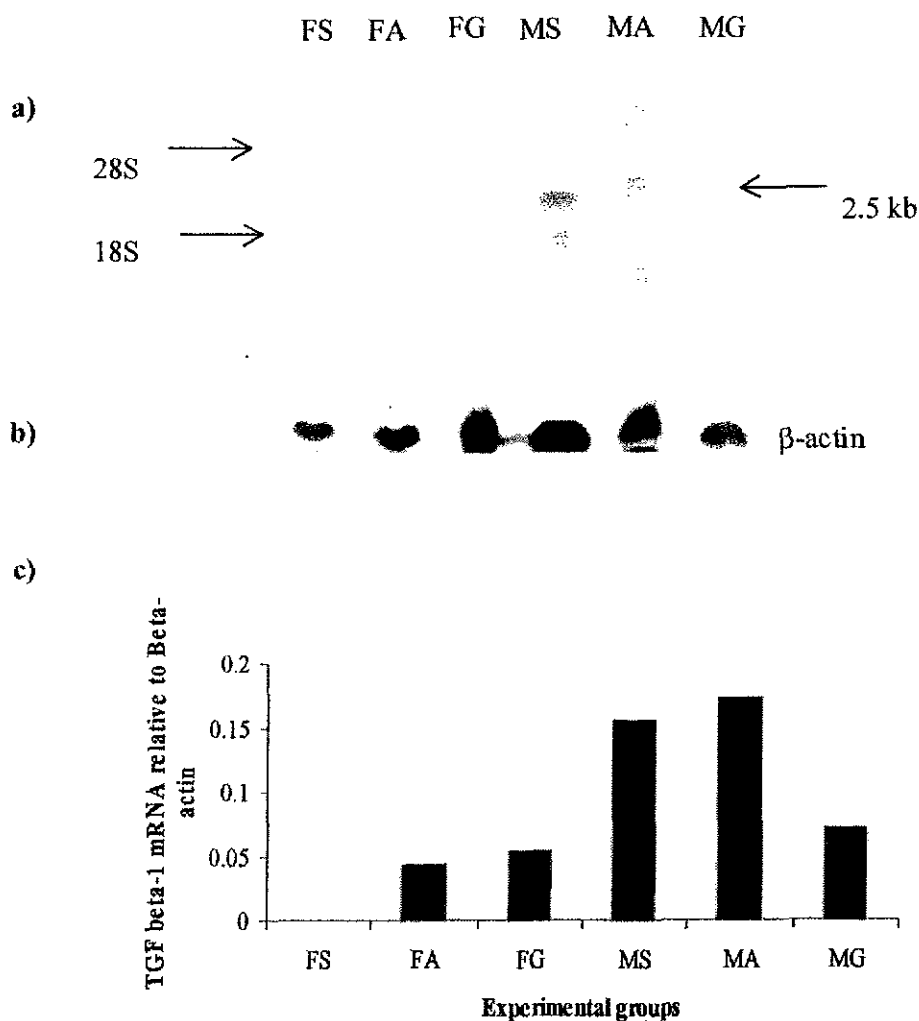


Figure 8.1.3; Expression of mRNA for TGF- β 1 was increased by Serine/Threonine and Glycine in female mice. In male, expression of mRNA was decreased by Glycine

FS = Female injected with saline

FA = Female injected with a mixture of Serine and Threonine

FG = Female injected with Glycine

MS = Male injected with saline

MA = Male injected with a mixture of Serine and Threonine

MG = Male injected with Glycine

18S and 28S=Indicating size of ribosomal RNA's which is a characteristic of nucleated cell (S=Svedberg unit).

KB= Indicating the size of mRNA (kilo base)

The injection of Serine/Threonine or Glycine did not influence mRNA expression for TGF- β_2 in the female but its expression was increased in the male. In the male, the injection of Serine/Threonine increased the expression of mRNA for TGF- β_2 more than the injection of Glycine (Figure 8.1.4).

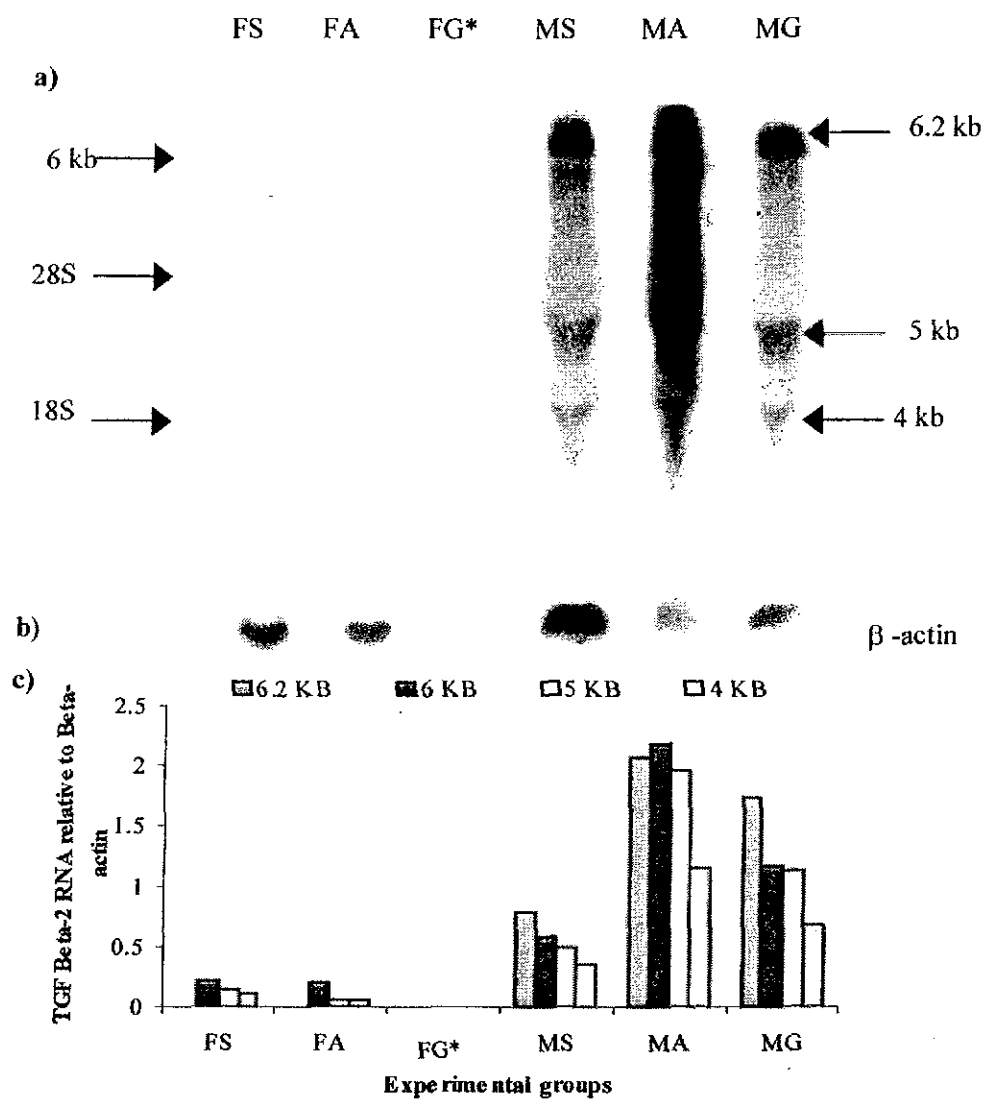


Figure 8.1.4.; Four different, 6.2kb, 5kb and 4kb of mRNA species for TGF- β_2 were found in mice testis and ovary. The expression of mRNA for TGF- β_2 was not effected by Serine/Threonine (A) and Glycine (G) in female mice (F) but, it was increased in male mice (M). The star indicates the absence of RNA on the blot.

The expression of mRNA for TGF- β_3 was increased by amino acid injection both in testis and ovary. The effect of Serine/Threonine was very large in the female (Figure 8.1.5).

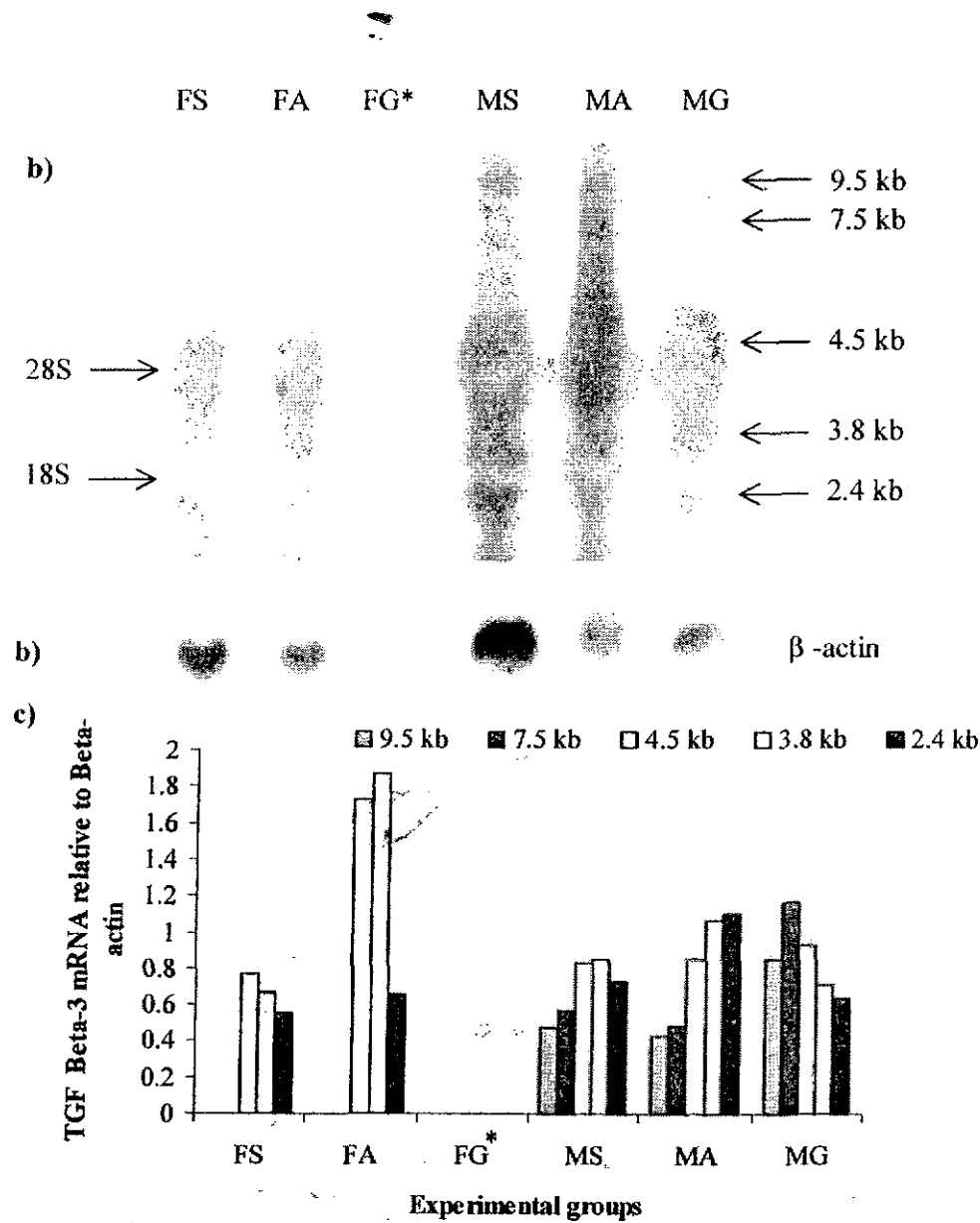


Figure 8.1.5; The injection of Serine/Threonine caused an increase in mRNA expression in female mice (FA). There was no RNA available for hybridisation from Glycine injected female mice (indicated with star). In male mice, mRNA expression for TGF- β_3 was increased by Serine/Threonine(MA) and Glycine (MG).

Both FSHR and LHR mRNA expressions, within the ovary were, decreased by amino acid injection whereas they were increased in the testis (Figure 8.1.6 and Figure 8.1.7).

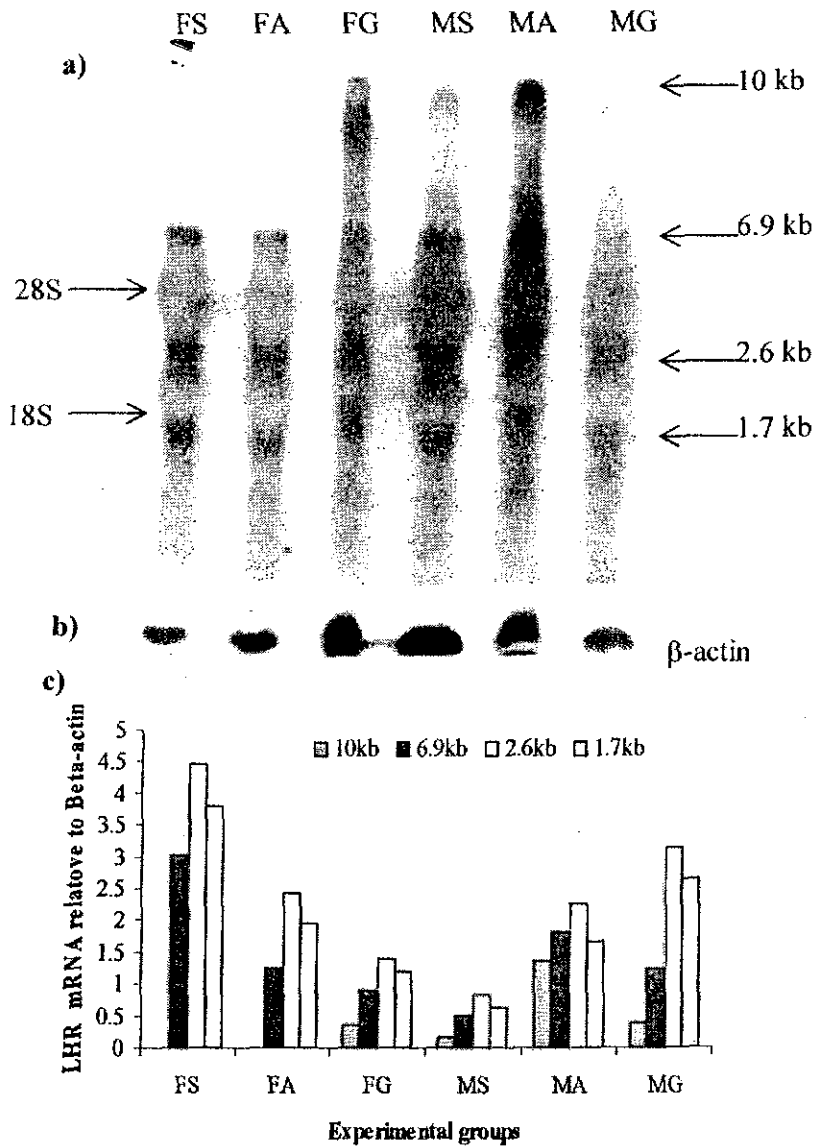


Figure 8.1.6; Expression of mRNA for LHR was decreased in the ovaries of Serine/Threonine (FA) and Glycine (FG) injected female mice but increased in the testis (MA and MG).

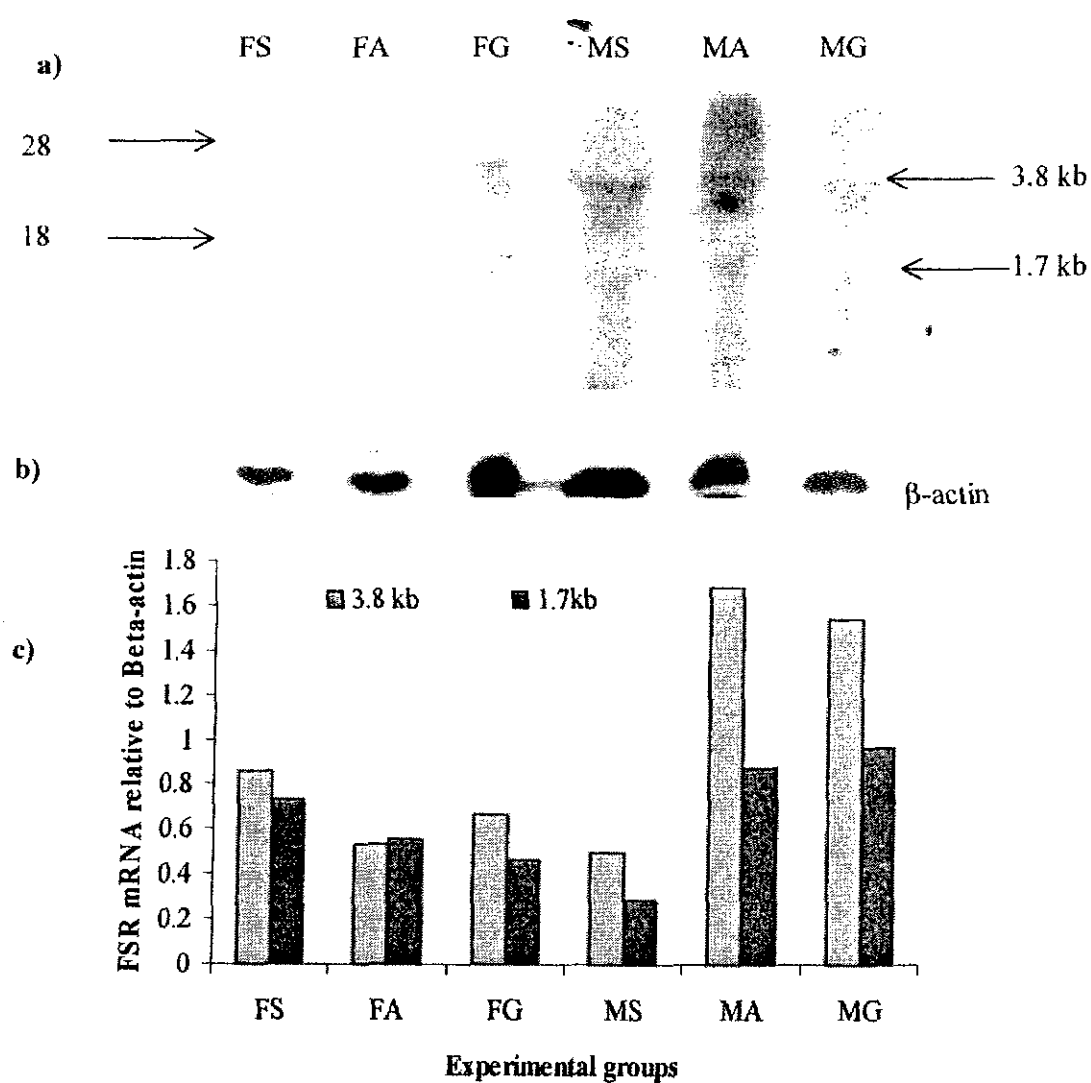


Figure 8.1.7; Expression of mRNA for FSHR was decreased by Serine/Threonine and Glycine injection in female mice (FA and FG). In male mice, expression of mRNA was increased by amino acid injection (MA and MG).

Expression of mRNA for P450_{scc} was decreased by amino acid injection in the female but it was increased in the male (Figure 8.1.8). Aromatase enzyme expression was decreased in the female whereas it was increased in the male by Serine/Threonine and Glycine injections (Figure 8.1.9).

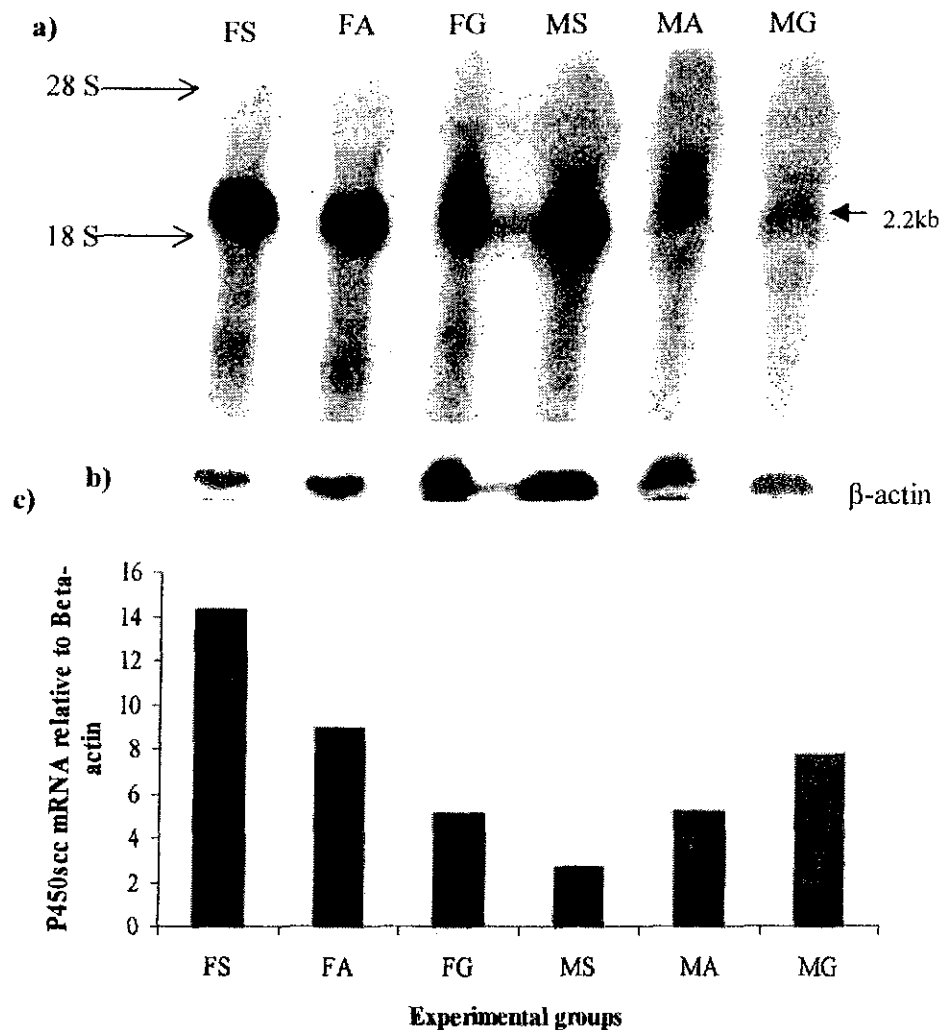


Figure 8.1.8; There was only one type of mRNA for side chain cleavage enzyme in mice testis and ovary. Its expression was decreased by amino acid injection in female mice, while it was increased in male mice (MA and MG).

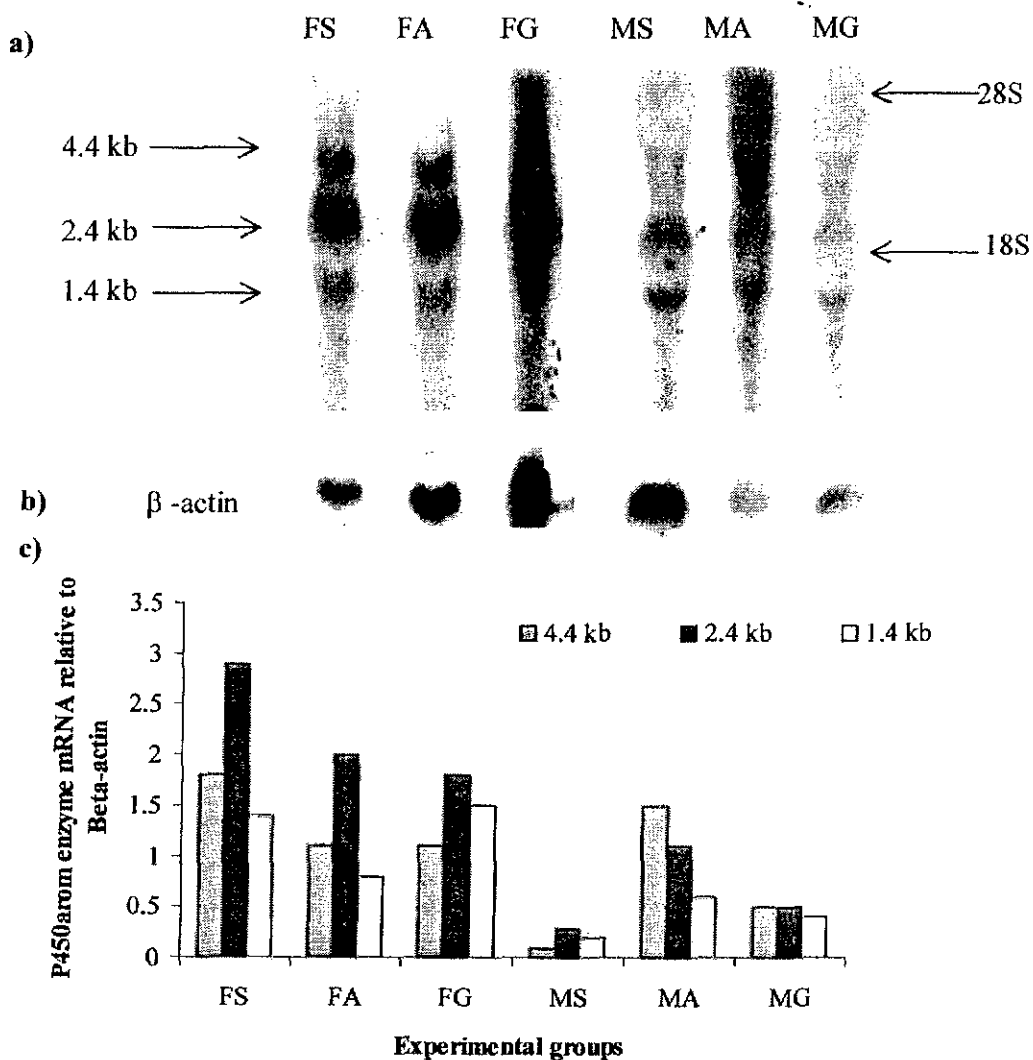


Figure 8.1.9; Serine/Threonine injection reduced the expression of aromatase enzyme within the ovary but in the testis, its expression was increased by amino acids.

DISCUSSION

The expression of mRNA for TGF- β_1 was tended to increase by the injection of a mixture of Serine and Threonine in both male and female mice. In male mice, TGF- β_1 is produced by Sertoli cells, Leydig cells (Lejeune *et al.*, 1996) and peritubular cells (Avallet *et al.*, 1994). In female mice, it is produced by theca, granulosa and interstitial cells (Engelhardt *et al.*, 1992; Mamluk *et al.*, 1998). It is secreted in a latent form (Graycar *et al.*, 1989). Expression of mRNA for TGF- β_1 is under the control of gonadotrophins and steroids. Its expression, in Leydig and Sertoli cell, is decreased by FSH and testosterone. This protein plays an autocrine and a paracrine role in the regulation of both ovarian and testicular function. Transforming Growth Factor- β_1 will inhibit LH-induced testosterone production by Leydig cell from 16.5 and 20.5 days old rat fetuses. This effect of TGF- β_1 is dose and time dependent (Gautier *et al.*, 1997). It has been reported that treatment of Leydig cells with high doses (10ng/ml) of TGF- β_1 inhibited LH-induced testosterone secretion while small doses (0.06-0.4 ng/ml) had positive effects (Benahmed *et al.*, 1989). The inhibitory effect of higher doses may be the result of decreased pregnenolone formation because of the decrease in LH-induced cAMP formation (Avallet *et al.*, 1987; Lin *et al.*, 1987) rather than because of alterations in the activity of steroidogenic enzymes *per se* (Benahmed *et al.*, 1989; Gautier *et al.*, 1997). Gonadotrophin induced aromatase activity was inhibited by TGF- β_1 . Treatment of porcine Sertoli cell with TGF- β_1 inhibited FSH induced aromatase activity (Moreara *et al.*, 1992). According to the literature, the primary action of TGF- β_1 is distal to cAMP formation and neither P450arom. nor P450scc are primary targets for TGF- β_1 (Engelhard *et al.*, 1992). The addition of TGF- β_1 to porcine Leydig cells in culture reduced the number of LH-R without modification of their binding affinity (Avallet 1987). In this experiment, mRNA expression for P450 arom. and P450 scc enzymes were both decreased in the female but increased in the male by Serine/Threonine injections. We hypothesise that the expression of TGF- β like peptides would increase following Serine and Threonine injection. The results presented here partly confirm this. However it seems that the expression of mRNA for TGF- β 's is more complex than we hypothesised. Both in male and female mice, the expression of mRNA for TGF- β_1 and β_3 was increased by Serine and Threonine

injections. In female mice, the injection of Serine and Threonine had no effect on expression of mRNA for TGF- β_2 , whereas it was increased in male mice. This result shows that, the expression of TGF- β like peptides can be increased by the injection of a mixture of Serine and Threonine. In female mice, expression of mRNA for the LH-R and FSH-R were both decreased by Serine and Threonine injection, whereas both were increased in male mice. Decreased expression of gonadotrophin receptors and steroidogenic enzyme suggest that Serine and Threonine facilitates atresia through increased expression of TGF- β like factors within the ovary. TGF- β probably causes luteinisation of follicular cells and increases androgen productin.

Another reason for the decreased expression of P450scc and P450arom expression might be the use of PMSG. Pregnant mare serum gonadotrophin has the combined actions of both LH and FSH. It has been reported that the addition of LH to luteinized bovine theca or granulosa decreased the expression of mRNA for P450scc enzyme (Mamluk *et al.*, 1998). Therefore, PMSG treatment might have decreased the expression of these enzymes in the mouse ovary. In the testis the expression of FSH-R, LH-R, P450scc and P450arom. were all increased by Serine and Threonine. If the TGF- β like factors have no effect on steroidogenic enzyme function, why was the expression of mRNA's for these enzymes decreased in the female mouse? This question can be answered by working with monolayer cultures of cells from the ovary or testis and adding TGF- β , Serine/Threonine and then measuring P450arom, P450scc, cAMP, testosterone and progesterone production. Avallet *et al.*, (1987) reported that TGF- β inhibited LH-R formation by pig Leydig cell *in vitro*. If the expression of TGF- β like factors was increased in the testis by Serine and Threonine, expression of gonadotrophin receptors should have decreased but, we observed the opposite effect. The effect of TGF- β on gonadotrophin receptor expression depends on the dose; high doses (10ng/ml) have inhibitory effect whereas lower doses are stimulatory (Engelhardt *et al.*, 1992). Probably, TGF- β is expressed in a small quantity in the testis. It is also possible that the effects of TGF- β like factors will depends on cell type. Because testicular cells have a paracrine effect within the testis. Sertoli and Leydig cell interaction increases LH-R number and steroidogenic potential within the testis (Benahmed *et al.*, 1986). We used mice and worked on the whole ovary and testis and that is the reason why we have slightly different results from others.

CHAPTER IX; GENERAL DISCUSSION

Serine/Threonine phosphorylation is an essential event for the regulation of cellular processes. Cell signalling, expression of new proteins, mitosis and cell cycle are all regulated by phosphorylation of Serine/Threonine. Phosphorylation of Serine/Threonine in target proteins anchored to the plasma membrane as well as in proteins in the cytosol modulate the signalling ability of hormones and growth factors (Hu *et al.*, 1997). Phosphorylation of Serine or Threonine at different parts of the protein modulates the function of growth factors (Luo and Lodish, 1997).

Nutrition can effect reproduction with specific nutrients (eg. amino acids) having roles in the regulation of neural systems related to reproduction or in biochemical processes within the tissues leading to the expression of new genes that effect growth and/or differentiation. It was hypothesised that supplementation with Serine/Threonine may increase phosphorylation of Serine/Threonine residues within the intra-cellular proteins and might stimulate new-high affinity binding sites for other intracellular signalling proteins required for downstream signalling, possibly TGF- β like peptides to modulate gonadotrophin action, cellular growth and hormone secretion in the ovary and testis.

During ovarian folliculogenesis, follicles undergo growth and maturation. Beyond the primordial stage, granulosa cells undergo active proliferation and differentiation until ovulation. The proliferation of granulosa cells is necessary for follicular growth (Zhang *et al.*, 1988). The growth and differentiation of follicles is under the control of factors (e.g. gonadotrophins) originating outside the ovary and other factors (e.g. growth factors, cytokines and steroids) produced within the ovary. Serine and Threonine are taken up by intracellular proteins and phosphorylated. Proteins taking part in gene expression and steroidogenesis are phosphorylated on Serine and/or Threonine. Treatment of Leydig cell with non-phosphorylatable analogues of Serine and Threonine reduced secretion of progesterone by decreasing activity of P450_{scc} enzyme (Stocco and Clark, 1993). It was thought that supplementation with Serine/Threonine might change the expression of TGF- β like factors through an effect on phosphorylation that in turn might effect granulosa cell proliferation and

differentiation. Therefore, pre-antral follicles from prepubertal mice were cultured in the presence of Serine/Threonine to measure the effect of amino acid supplementation on follicular growth (Chapter 3). Male and female mice were injected with Serine/Threonine for 5 days and ovaries or testes were removed for histological examination to study follicle growth, differentiation and testis function.

Pre-antral follicles growth was slightly more rapid in mice injected with amino acids but the effect was not significant. Transforming Growth Factor- β like factors are expressed in the theca, granulosa and oocyte of mature mouse follicles (Ghiglieri *et al.*, 1995). Experiments on pigs show that, the secretion of TGF- β like factors was increased significantly as follicles grew through the preantral and antral stages (May *et al.*, 1996). Transforming Growth Factor- β can stimulate preantral follicle growth and this effect is age dependent. In adult mice, TGF- β caused a significant increase in follicular growth and inhibin production in a dose dependent manner, but the size of follicles, from immature mice (11 days old), did not change after TGF- β administration (Liu *et al.*, 1999). The growth of preantral follicles is not dependent on gonadotrophins. Preantral follicles can initiate their own growth even in the absence of gonadotrophins and TGF- β is probably involved with this growth process (Liu *et al.*, 1999). Our results from gene expression studies (chapter 8) show that expression of TGF- β_1 and TGF- β_3 increased in ovaries of mice injected with Serine and Threonine and this probably facilitated the growth of preantral follicles. However, Liu *et al.*, (1999) reported that TGF- β like factors have no influence on the growth of preantral follicles of immature mice, note that we used 19-21 days old mice.

The results from testis histology (chapter 5) showed that the number of spermatids per testis slightly reduced (not significantly) by Serine/Threonine injections. This may be the result of increased expression of TGF- β_1 , TGF- β_2 and TGF- β_3 within the testis. The lower number of spermatids might also be the result of inhibition of Leydig cell function caused by increased expression of three species of TGF- β in the testis of mice injected with Serine/Threonine. It has been reported that TGF- $\beta_{1,2}$ and 3 decreased testosterone production and the number of spermatids in the testes of adult mice (Ohta *et al.*, 1996) and in fetal testis of fetal Wistar rats (Olaso *et al.*, 1998). This reduction in the number of spermatids was attributed directly to the increase in

apoptosis of gonocytes without changing their mitotic activity during the developmental phases of proliferation (Ohta *et al.*, 1996; Olaso *et al.*, 1998).

High level of intra-testicular testosterone is a prerequisite for the maintenance of spermatogenic function. Treatment of hypogonadal mice with testosterone alone did not disrupt spermatogenesis. Even small dose of testosterone is enough for the maintenance of spermatogenesis. It was reported that testosterone alone could induce spermatogenesis in the absence of gonadotrophins (Singh and Handelsman, 1996).

Gonadotrophic stimulation of testis is important for production of appropriate numbers of spermatozoa (Zirkin, 1998). Experiment on hypogonadal mice shows that FSH increases mitosis of spermatogonium and spermatocytes and treatment together with FSH and testosterone further increased mitosis of germ cell (Singh and Handelsman, 1996). It has been reported that TGF- β s produced within the testis inhibit function of pig Leydig cell by decreasing testosterone and cAMP response to LH without any modification of binding affinity (Avallet *et al.*, 1987; Lejeune *et al.*, 1996). Therefore we suggest the reason for reduced number of spermatids is not a result of reduction in testosterone secretion due to the increased expression of TGF- β like factors within the testis in response to Serine and Threonine injection.

In female mice, the expression of FSHR was decreased by amino acid injection. Our results show that, Serine/Threonine injection increased the expression of TGF- β 1 and TGF- β 3 within the ovary of mice. This might apply to sheep as well. Serine/Threonine injections may have caused increased production of TGF- β which suppressed of FSH secretion via an increase in inhibin production. Treatment of rat granulosa cells with TGF- β increased FSH-induced inhibin production (Zhang *et al.*, 1988). Addition of inhibin (extracted from porcine follicular fluid) has been reported to inhibit secretion of FSH from rat pituitary cells *in vitro* (Ying *et al.*, 1986).

There is also a possibility that Serine/Threonine injection may have had a direct effect on hypothalamo-pituitary axis affecting secretion of gonadotrophins. Our results from the sheep work show that secretion of FSH was suppressed while the secretion of LH was increased. More work is necessary to find the reasons behind this and to confirm whether Serine/Threonine injection directly acts on ovary or hypothalamus and pituitary. This could be done by the injection of ovariectomised sheep with Serine/Threonine and monitoring secretion of gonadotrophins. To measure the effect

of Serine/Threonine injection on the pituitary, *in vitro* work is required using pituitary cell culture and adding amino acids and GnRH to test for differences in gonadotrophin secretion. The effect on LH and FSH could be an indirect effect of Serine/Threonine injection acting through the inhibin production within the ovary to suppress pituitary FSH secretion. To find out if there is an effect of Serine/Threonine injection on inhibin expression within the ovary, experiments in normal sheep are required to confirm increased inhibin secretion. It is also important to find out in which particular cell type within the ovary and testis the expressions of TGF- β were increased. Therefore, *in situ* hybridisation work must be conducted to show differences in tissue specific expression.

The result from ovarian histology shows that antrum formation was increased by amino acid injection. In the preantral stage more follicles became atretic in the ovaries of Serine/Threonine injected mice. Atresia in antral follicles decreased in test group. Antral follicles expressed TGF- β like factors and the expression of mRNA increased during development. Both the theca and the granulosa of non-atretic antral follicles, from porcine ovaries, expressed mRNA for TGF- β like factors whereas, the expression of mRNA in theca of atretic antral follicles was noticeably absent although some residual expression was found in granulosa of atretic antral follicles (May *et al.*, 1996). The decrease in atresia in the antral stage might be due to an increase in the expression of mRNA for TGF- β like factors within the ovary by amino acid injection. Injection of Serine/Threonine increased the expression of TGF- β 1 and β 3 but decreased the expression of gonadotrophin receptors and expression of P450arom and P450scc enzyme. The function of TGF- β like factors within the ovary depends on the species. For instance, the pig, TGF- β 1 and β 2 inhibit FSH-dependent LH receptor induction, whereas in rat they potentiated FSH-dependent LH receptor induction (Gitay-Goren *et al.*, 1993). Therefore, increased atresia could be a result of decreased expression of receptors for LH and FSH. Formation of gonadotrophin receptors with normal function takes 3-4 days in mice follicles *in vitro*. Down regulation of the receptor takes a shorter time than for formation of that receptor. At the gene level, it is extremely short and the receptor is expressed or suppressed within hours. We injected mice for five days and that was enough time to suppress expression of the gene receptor. Increased atresia in the test mice is also a possible result of decreased

steroidogenic enzyme expression. In amino acid injected mice, preantral follicle growth increased. It is also possible that increased atresia in test group was due to a higher number of growing preantral follicles. When there is a high number of follicle growing, the incidence of atresia will increase. Because atresia mostly occurs in preantral stage. Probably, follicles in preantral stage are more susceptible to atresia than large follicles.

Expression of genes for P450arom and P450scc enzymes are important for progesterone and oestradiol biosynthesis. The data from last experiment showed that the expression of these enzymes increased in parallel with the expression of gonadotrophin receptors in the male whereas they were decreased in the female by amino acid injections. This could be another reason for the increased atresia of preantral follicles in test animals. Addition of FSH and LH to mature granulosa cells from the human, stimulated the expression of mRNA for P450arom. Stimulation of human granulosa cells with LH and FSH also increased the expression of P450scc (Yong *et al.*, 1994). If there is a decrease in the expression of the receptor for gonadotrophins it means the response of the follicles to the gonadotrophins is decreased causing a decrease in steroidogenic enzyme gene expression within the ovary. In the testis, expression of these enzymes was increased as was the expression of LH and FSH receptors. Amino acid injection caused an increase in the expression of FSR in the testis and this probably led to an increase in steroidogenic enzyme expression. Treatment of pig Sertoli cells with FSH increased aromatase activity in a dose dependent manner. Addition of TGF- β 1 (2ng/ml) inhibited FSH-induced aromatase activity by reducing FSH-stimulated cAMP production through the catabolism of the cyclic-nucleotides by increasing cAMP-phosphodiesterase activity (Morera *et al.*, 1992).

The data we have presented show that expression of TGF- β 1, β 2 and β 3 was upregulated in test groups over the control but not dramatically and these increases are not capable of producing inhibitory actions on FSH-induced aromatase activity but, these increases are able to reduce the number of spermatids in the testis.

CHAPTER X; GENERAL CONCLUSION

The objective of this work was to test the effect of injecting a mixture of Serine and Threonine on follicular growth, hypothalamo-pituitary function, expression of genes for TGF- $\beta_{1,2}$ and β_3 , follicular differentiation and atresia (mice or sheep were injected with a mixture of Serine and Threonine and the effect on LH and FSH secretion, the expression of genes for LH and FSH receptors, genes for TGF- $\beta_{1,2}$ and β_3 and the genes for steroidogenic enzymes were measured). Six experiments were conducted. An *in-vitro* study was conducted (chapter 3) to measure the effect of a mixture of Serine and Threonine on pre-antral follicle growth in terms of changes in diameter. Two *in vivo* experiments were conducted to measure the effect of a injecting a mixture of Serine and Threonine on ovary and testis function by means of their effects on follicle growth, differentiation, atresia, seminiferous tubule diameter, the diameters of Leydig cells and the number of spermatids (chapter 4 and 5). Two *in vivo* studies were carried out to test the combined effect of these amino acids on LH and FSH secretion in sheep. An *in vitro* experiment was conducted to test the combined effect of these amino acids on the expression of the genes for LH and FSH receptors, steroidogenic enzymes (P450arom and P450scc) and genes for TGF- $\beta_{1,2}$ and β_3 in the gonads of mice.

Preantral follicle growth and antrum formation were increased by the injection of a mixture of Serine and Threonine (chapter 4) and this was associated with increased expression of mRNA's for TGF- β_1 and β_2 (chapter 8). The injection of Serine and Threonine also increased the incidence of atresia in preantral and antral stage follicles. Increased expression of TGF- β 's in the ovary probably reduced the expression of mRNA's for gonadotrophin receptors and thus the expression of steroidogenic enzymes leading to increased atresia in preantral and antral stage follicles.

In the sheep decreased FSH secretion (chapter 7) is a result of increased oestradiol and/or inhibin production and these may be associated with increased TGF- β production. The increased LH secretion seen in sheep (chapter 6) indicates a possible effect of Serine and Threonine on the central nervous system and a role in regulating

the secretion of gonadotrophins. It is not certain that the decrease in FSH secretion is a result of increased inhibin production by the ovary. It could also be a direct effect of these amino acids on the GnRH secretory system, but Serine/Threonine injection, increased the secretion of LH whereas the secretion of FSH was decreased (chapter 7). This is probably because the secretion of LH and FSH are differentially regulated at the level of the pituitary gland.

The decrease in number of spermatids, in the testis (chapter 5) of Serine and Threonine injected mice, is not the result of disrupted hormonal balance, but probably the result of an indirect effect of Serine and Threonine on apoptosis of gonocytes in the testis again associated with increased expression of TGF- β like proteins (chapter 8). It appears to be similar to the effect on atresia that was seen in the ovaries of mice following the Serine and Threonine injection.

The data shows that injection of a mixture of Serine and Threonine caused a significant reduction in the number of follicles entering to primary stage and a non-significant increase in follicle growth (beyond the primary stage), antrum formation and atresia. Injection of Serine and Threonine caused a reduction in the number of spermatids, whereas the diameters of seminiferous tubules and the diameters of the Leydig cells were not changed by amino acid injection. Injection of Serine and Threonine significantly increased the secretion of LH, whereas the secretion of FSH was suppressed. Amino acid injection increased the expression of genes for TGF- β_1 , β_2 and β_3 . The expression of genes for steroidogenic enzymes decreased in female mice while they increased in male mice by amino acid injection.

These results shows that nutrition can exerts an effect on reproductive function of females and possibly also in male by means of amino acids (Serine and Threonine) which may effect intra-cellular biochemical processes important for gene transcription, hormone production and regulation of the function of the gonads.

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