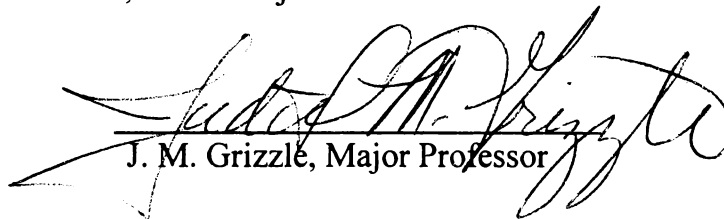


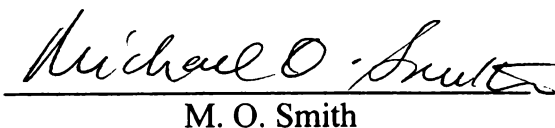
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I am submitting herewith a thesis written by Matthew D. Brent entitled "The effect of tumor necrosis factor alpha (TNF α) on *in vitro* steroidogenesis in two strains of White Leghorn hens." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.


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**THE EFFECT OF TUMOR NECROSIS FACTOR ALPHA (TNF α) ON *IN*
VITRO STEROIDOGENESIS IN TWO STRAINS OF WHITE LEGHORN HENS**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Matthew Daniel Brent
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ABSTRACT

Two strains of White Leghorn hens selected for high (HA) or low (LA) antibody response to challenge with sheep red blood cells were used to determine differences in circulating levels of progesterone (P_4) and luteinizing hormone (LH), and to determine *in vitro* effects of tumor necrosis factor alpha ($TNF\alpha$) on granulosa cell steroid secretion during different periods of the estrous cycle. In experiment one, hens were serially bled from 4 hours after oviposition until the next oviposition. Plasma was analyzed for P_4 and LH using validated radioimmunoassays (RIA's). In the second experiment, hens were sacrificed at 16 and 37 weeks of egg production. Granulosa cells isolated from the F_1 , F_2 , and F_3 follicles were incubated for 3 hours in the presence of; 1) untreated RPMI 1640 + 0.1% bovine serum albumin + 1% antibiotics (control) 2) 5 ng/ml human recombinant $TNF\alpha$ (h $TNF\alpha$) 3) 5 ng/ml murine recombinant $TNF\alpha$ (m $TNF\alpha$) 4) 100 ng/ml ovine LH (oLH) 5) h $TNF\alpha$ plus oLH 6) m $TNF\alpha$ plus oLH. Conditioned media was analyzed for P_4 and P_5 secretion as before.

Plasma levels of both P_4 and LH were significantly higher ($P \leq 0.05$) among HA hens during the peak preovulatory period of the estrous cycle (-6 to -1 hours before oviposition). Circulating levels of P_4 were 1.45, 2.19, 2.67, 2.66, and 2.38 ng/ml in LA hens versus 1.82, 3.05, 3.19, 3.07, 3.01, and 2.79 ng/ml respectively in HA hens at 6, 5, 4, 3, 2, and 1 hour prior to oviposition. Levels of LH were 2.36, 3.36, 3.57, 2.81, 2.41, and 1.95 ng/ml in LA hens versus 3.58, 5.27, 5.38, 4.51, 3.77, and 3.71 ng/ml respectively in HA hens for the same period.

As with plasma, granulosa cells from HA hens produced more ($P \leq 0.05$) P_4 and P_5 than did those from LA hens. Similarly, granulosa cells from larger follicles secreted more ($P \leq 0.05$) P_4 and P_5 than did those from smaller follicles. Treatment of granulosa cells with human, but not murine, $TNF\alpha$ reduced ($P \leq 0.05$)

basal P_4 and P_5 production by granulosa cells from preovulatory follicles. Conversely, treatment of cells with oLH significantly increased ($P \leq 0.05$) P_4 and P_5 as compared to controls. The combination of mTNF α , but not hTNF α , and oLH consistently stimulated ($P \leq 0.05$) P_4 and P_5 secretion above that observed with treatment of oLH alone. Mouse TNF α is known to activate both type I and type II TNF α receptors, while hTNF α binds only the type I receptor. This may explain the differential effects on *in vitro* steroid secretion observed through use of the two forms of TNF α in these experiments. Furthermore, the observation that hTNF α and oLH rarely caused significant increases, but frequently caused numerical increases in P_4 and P_5 secretion is suggestive of a ligand passing role for the type II receptor. No clear pattern emerged as to when during the estrous cycle hTNF α affected steroid production. In the avian, reduced steroidogenic capacity (specifically, the production of progesterone) can lead to delayed or inhibited ovulation. Results from these experiments suggest a potential role for human and/or murine TNF α in reproduction in the laying hen (*Gallus domesticus*).

1. LITERATURE REVIEW

Cytokines are a group of 6,000 - 60,000 kiloDalton (kDa) proteins, of which over thirty have been isolated since their discovery in the early 1970's (Rafael and Orvieto, 1992). These proteins are derived from activated immune cells and are involved in cell-to-cell communication, resulting in initiation of the immune response, inflammatory reactions, and hematopoiesis (Dinarello and Mier, 1987). These pleiotropic peptides are also potent regulatory factors. Unlike hormones, which are usually produced at a specific locus and act systemically, cytokines are produced by a wide variety of cells and act in an autocrine, paracrine, or endocrine fashion (Klasing and Johnstone, 1991). Cytokines can be further subgrouped into interleukins, lymphokines, and monokines. The term interleukin refers to soluble factors which mediate communication between leukocytes, while lymphokines and monokines designate cytokines which are produced by lymphocyte and monocyte/macrophage cells respectively (Rafael and Orvieto, 1992). As more information is compiled concerning their source and function, it is apparent that many cytokines fall into more than one of these subgroupings.

Tumor necrosis factor alpha (TNF α) is a potent monokine that was discovered in 1975 in an attempt to identify a protein involved in tumor cytotoxicity (Carswell *et al.*, 1975). TNF α is also referred to as “cachectin” for its involvement in the phenomenon of wasting, and “necrosin” for its ability to cause cell death (Beutler and Cerami, 1986). TNF α is classified as a type II membrane protein which is characterized by a C-terminus in the extracellular domain. Other type II membrane proteins include; TNF β (lymphotoxin), Fas ligand, OX40 ligand, Chain of Differentiation (CD) 40 ligand, CD27 ligand, CD30 ligand, and lymphotoxin β (Gruss and Dower, 1995). Structurally, TNF α is a 26 kDa membrane bound protein which has a 29 amino acid (aa) cytoplasmic tail, a 28 aa transmembrane region, and a 176 aa extracellular domain (Gruss and Dower, 1995). Proteolytic cleavage of the membrane bound protein by TNF α converting enzyme (TACE) results in the release of a 17.4 kDa, 157 aa soluble protein as a homotrimer with a three dimensional structure similar to the “jelly roll” motif shared by various viral capsids (Kreigler *et al.*, 1988; Brännström *et al.*, 1993; Black *et al.*, 1997). TNF α is expressed by many cell types including macrophage, CD4 $^{+}$ and CD8 $^{+}$ thymocyte (T) cells, keratinocytes, adipocytes, kidney tubule epithelial cells, splenocytes, astrocytes, pancreatic β -cells, neurons,

and osteoblasts (Hunt *et al.*, 1992; Ware *et al.*, 1992; Lee *et al.*, 1993; Yamada *et al.*, 1993; Kern *et al.*, 1995; Lisby *et al.*, 1995; Modrowski *et al.*, 1995; Villarroya *et al.*, 1997). Due to the wide variety of cells which express TNF α , it is more practical to list those cell types in which this cytokine is not expressed or has yet to be identified. These include erythrocytes and resting T cells which do not express the protein nor its receptors (Fiers, 1991). Both human and mouse TNF α have been cloned and exhibit approximately 79% amino acid sequence homology (Fransen *et al.*, 1985; Pennica *et al.*, 1985). Experimental evidence suggests that normal circulating levels of TNF α are between 10 and 80 pg/ml (Steinshamn *et al.*, 1995; Spengler *et al.*, 1996). Beutler *et al.* (1985) determined the half-life of TNF α in plasma to be approximately six minutes.

To date, two TNF α receptors have been identified, type I and type II. Both receptors belong to the TNF α /nerve growth factor receptor (NGFR) superfamily and share 28% sequence homology in their extracellular domains (Tartaglia *et al.*, 1993). Their intracellular domains, however, apparently share no sequence homology, suggesting the use of different signaling pathways (Lewis *et al.*, 1991). The TNF α receptors are classified

as type I membrane proteins characterized by 4 cysteine-rich motifs in the extracellular binding domain (Baker and Reddy, 1996). Both receptors can be released as soluble proteins, via proteolytic cleavage of their extracellular domains. Soluble receptors have been identified in the serum and urine of normal and diseased patients (Spengler *et al.*, 1996; Olsson *et al.*, 1989). Physiologically, soluble receptors may act to inhibit TNF α activity, as cleavage of the extracellular domain reduces the number of cell surface receptors. Soluble receptors may also bind to soluble TNF α , thereby further reducing concentrations of the cytokine available for receptor binding at the cell surface.

The type I TNF α receptor has a molecular weight of 55 kDa, and is designated TNF-R1, TNF-R55, p55, or CD120a (Fiers, 1991). This glycoprotein, consisting of a 190 aa extracellular region, a 25 aa transmembrane segment, and a 220 aa cytoplasmic domain, binds to the human and murine forms of TNF α with equal affinities (Gruss and Dower, 1995; Lewis *et al.*, 1991). Studies utilizing monoclonal and polyclonal antibodies directed against TNF-R1 identify it as the receptor responsible for the majority of actions associated with TNF α . These studies implicate the type I receptor in cytotoxicity, fibroblast proliferation, resistance to

Chlamydia trachomatis, antiviral activity, and synthesis of prostaglandin E₂ (Englemann *et al.*, 1990; Espevik *et al.*, 1990; Shalaby *et al.*, 1990; Wong *et al.*, 1992). Tartaglia *et al.* (1993) identified a region of approximately 80 aa's within the intracellular domain of the type I receptor that is responsible for initiating the signal for cytotoxicity.

The type II TNF α receptor has a molecular weight of 75 kDa and is referred to as TNF-R2, TNF-R75, p75, or CD120b (Fiers, 1991). This glycoprotein consists of a 240 aa extracellular domain, a 27 aa transmembrane segment, and a 173 aa cytoplasmic domain (Gruss and Dower, 1995). The larger, type II receptor exhibits a 5 fold greater affinity for TNF α than does the type I receptor (Barbara *et al.*, 1994). Unlike TNF-R1 which binds to both human and murine TNF α , the type II receptor binds only to murine TNF α (Lewis *et al.*, 1991). Monoclonal and polyclonal antibody studies reveal that activation of this receptor stimulates proliferation of primary thymocytes and cytotoxic T-cells (Sheehan *et al.*, 1995; Tartaglia *et al.*, 1991).

Biological effects of TNF α on various cells and tissues have been studied extensively. TNF α blocks the synthesis of triglycerides while concurrently stimulating adipocytes to release current stores into the plasma,

thus implicating TNF α in the phenomenon known as “wasting” (Beutler and Cerami, 1988). Complicating this already dangerous pathological state, TNF α activates osteoclasts causing the subsequent resorption of bone (Bertolini *et al.*, 1986). Furthermore, Garrett *et al.* (1987) showed that TNF α inhibited cartilage synthesis while promoting proteoglycan breakdown.

In addition to its involvement in cachexia, TNF α is thought to be responsible for many of the symptoms which accompany endotoxic shock, including; fever, hypotension, diarrhea, metabolic acidosis, and in severe cases, death (Beutler and Cerami, 1986). TNF α has also been observed to cause fever by inducing hypothalamic neurons to produce prostaglandin E₂ (PGE₂) (Dinarello *et al.*, 1986).

However, TNF α is best known for its involvement in the inflammatory reaction of the immune response. TNF α enhances immune responses via activation of neutrophils and eosinophils (Silberstein *et al.*, 1986). Neutrophil adhesion, phagocytosis, and complement receptors are reportedly increased upon exposure to TNF α (Shalaby *et al.*, 1985; Klebanoff *et al.*, 1986; Berger *et al.*, 1988). The proposal that ovulation is

akin to an inflammatory reaction suggests the possibility that cytokines may play a role in reproductive function (Espey, 1980).

Several studies have indicated the presence of TNF α within the ovary of various species. Using immunocytochemistry, immunoreactive TNF α has been localized in oocyte, granulosa, and mononuclear cells within the ovaries of the mouse and rat (Chen *et al.*, 1993; Marcinkiewicz *et al.*, 1994; Roby and Terranova, 1989). In humans, immunoreactive TNF α has been identified in granulosa, luteal, and paraluteal cells of the ovary (Roby *et al.*, 1990). Evidence also exists for biologically active TNF α in cumulus cells of the oocyte in the human, as well as follicular fluid in the human and bovine (Zolti *et al.*, 1990; Zolti *et al.*, 1991; Wang *et al.*, 1992; Jasper and Norman, 1995). Naylor *et al.* (1990; 1993) determined the presence of TNF α mRNA in human thecal cells via *in situ* techniques. In the corpus luteum, TNF α has been identified in endothelial cells and large luteal cells of swine (Hehnke-Vagnoni *et al.*, 1995; Wuttke *et al.*, 1993). Similarly, TNF α has been identified in the corpus luteum of the rabbit, sheep, and cow (Bavangados *et al.*, 1988; 1990; Ji *et al.*, 1991; Shaw and Britt, 1995).

Although the presence of TNF α in these tissues is readily accepted, its origin remains to be elucidated. Bagavandoss *et al.* (1990) proposed that

the primary source of TNF α was from extraovarian white blood cells infiltrating the ovary; while others suggest that TNF α may be derived from cells resident in the ovary itself (Sancho-Tello *et al.*, 1992). The findings by Zolti *et al.* (1990) that human granulosa cells are both a source and target for TNF α lend credence to the theory that there is an intraovarian source of TNF α , and that it may play a role in the reproductive cycle.

In the growing mammalian follicle, granulosa cells are responsible for the production of estrogen, as well as a small amount of progesterone. In several studies, TNF α has been shown to suppress either aromatase or estradiol production by granulosa cells. Emoto and Baird (1988) reported that in cultured rat granulosa cells, 10 pg/ml - 10ng/ml TNF α suppressed aromatase activity in the presence of 250 ng/ml follicle stimulating hormone (FSH). This dose-dependent response culminated in the total abolition of FSH induced aromatase activity at 10 ng/ml TNF α . A time-course study revealed that TNF α did not inhibit the activity of the existing enzyme, but rather, blocked any subsequent induction of aromatase activity by FSH. Similarly, Adashi *et al.* (1990) showed a significant reduction in FSH induced estradiol production in rat granulosa cells (10 ng/ml TNF α). In small human and bovine follicles, TNF α inhibited FSH induced aromatase

activity and estradiol production by granulosa cells (Montgomery *et al.*, 1996; Spicer and Alpizar, 1994). Furthermore, TNF α (0.1-100 ng/ml) was shown to reduce FSH induced LH receptor formation in cultured rat granulosa cells, with an almost complete inhibition at 50 ng/ml (Darbon *et al.*, 1989). Taken together, these findings suggest a possible role for TNF α in modulation of granulosa cell differentiation.

In response to an LH surge, mammalian granulosa cells luteinize to become granulosa-luteal cells, and eventually, either small or large luteal cells of the corpus luteum. These cells are responsible for the increased progesterone production associated with the luteal phase of the estrous cycle and pregnancy. Adashi *et al.* (1990) reported that TNF α (10 ng/ml) caused significant inhibition of FSH stimulated (100 ng/ml) progesterone production in granulosa-luteal cells of the rat. Similarly, TNF α caused decreased production of progesterone in response to LH, oxytocin, and forskolin in the small luteal cells of swine (Richards and Almonds, 1994; Wuttke *et al.*, 1993). TNF α reportedly increased the production of prostaglandins, specifically PGF_{2 α} , in luteal cells of the human and bovine, as well as in luteinized granulosa luteal cells in the human (Wang *et al.*, 1992; Zolti *et al.* 1990; Benyo and Pate, 1992). The ability of TNF α to

inhibit gonadotropin induced progesterone production, while stimulating that of prostaglandins, suggests a luteolytic role for the cytokine (Pate, 1995).

In mammals, the granulosa cell layer is responsible for the production of estrogens, whereas the theca layer contains androgen and progesterone producing cells. Roby and Terranova (1990) found that basal and FSH induced progesterone were inhibited by $\text{TNF}\alpha$ in rat granulosa-luteal cells, however, in cultured whole follicles (theca included) under the same conditions, progesterone production was actually increased by $\text{TNF}\alpha$, with a concurrent increase in androgen production by the theca cells (Roby and Terranova, 1990). This implies the possibility that the effects of $\text{TNF}\alpha$ on steroid production in isolated granulosa cells may be very different from its effects on granulosa cells co-cultured with theca cells. Conversely, in the rat, $\text{TNF}\alpha$ caused a dose-dependent inhibition of human chorionic gonadotropin-stimulated androsterone and androstenedione production in whole ovarian dispersates (Andreani *et al.*, 1991; Zachow *et al.*, 1992). In theca cells of the pig, $\text{TNF}\alpha$ caused a dose-dependent inhibition of LH stimulated progesterone at concentrations of between 0.1 and 100 ng/ml (Tekpetey *et al.*, 1993). Interestingly, $\text{TNF}\alpha$ has been shown to induce

clustering of theca-interstitial cells of the rat (Zachow *et al.*, 1992). It has therefore been suggested that TNF α may play some role in the early organization of follicular cells (granulosa and/or theca) around the oocyte (Terranova *et al.*, 1995).

While the effects of TNF α on ovarian steroidogenesis may be apparent, the mechanism(s) by which they are brought about are not. Adashi *et al.* (1989) ruled out the possibility that the inhibitive action of TNF α on granulosa cell steroidogenesis is a lasting nonspecific effect due to its cytotoxicity. TNF α does not significantly reduce cell number, DNA content, or viability, and the effects of TNF α are reversed upon removal of the cytokine (Adashi *et al.*, 1989). These data suggested that TNF α directly and specifically regulates steroidogenesis. Luteinizing hormone receptor numbers are reportedly reduced by TNF α in the follicular cells of the rat (Darbon *et al.*, 1989). Tumor necrosis factor α has been reported to inhibit LH induced cAMP accumulation in theca and granulosa cells of the pig and rat (Tekpetey *et al.*, 1993; Veldhuis *et al.*, 1991; Zachow *et al.*, 1992; Emoto and Baird, 1988). Zachow *et al.* (1993) found that in addition to LH receptors and cAMP, TNF α directly inhibited protein kinase A. Tumor necrosis factor α also inhibited the activities of P450 side chain cleavage

(P_{450scc}) and 3 β -hydroxysteroid dehydrogenase enzymes, as well as the expression of cytochrome P_{450scc} mRNA in swine (Veldhuis *et al.* 1991).

Logically, one might assume the TNF α has the same effect on ovarian steroidogenesis in the avian as that in the mammal. However, the avian ovary differs from mammalian ovaries in a number of important ways. Initially, both left and right ovaries and oviducts are present in embryonic hens. However, shortly after hatching, the right ovary regresses in response to an unknown factor(s) secreted by the left ovary (Marrone and Hertendy, 1983). The majority of follicles on the ovary of the hen are small white or small yellow follicles (SWF's or SYF's) with diameters between 1 and 8 millimeters (mm; Armstrong, 1985). The larger (>8mm diameter), yolk-filled follicles, which are attached to the ovary by follicular stalks, are arranged in a hierarchy based on size (Bahr and Nalbanov, 1977). The largest of these five or six pre-ovulatory follicles (F₁, F₂,...F₆) will be the next to ovulate (Gilbert, 1971). When the largest follicle (F₁) ovulates, the former F₂ follicle moves up in the hierarchy (becoming the F₁ follicle), as do all other pre-ovulatory follicles. Unlike mammals, there is no corpus luteum (CL) formation following ovulation in the hen. However, the biggest difference is that granulosa cell progesterone is the predominant hormone

driving the avian cycle, rather than estrogen as in the mammal. These differences suggest that the role played by TNF α in reproductive function (if any) may be quite different for avian species.

As the follicle matures, the great increase in size is accompanied by changes in steroidogenic function. Small white follicles are the major source of estrogen in the hen (Armstrong, 1984). While the largest pre-ovulatory follicle (F₁) is known to be responsible for the majority of progesterone produced in the hen ovary, some contribution is made by the smaller pre-ovulatory follicles (F₂-F₄; Huang *et al.*, 1979). These differences are due to changes in granulosa and theca cell physiology as the follicle matures.

Each follicle on an avian ovary consists primarily of two cell layers. The innermost layer made up of granulosa cells which surround the yolk and ovum of the follicle. The theca layer, which is highly vascularized and innervated, is located immediately outside and adjacent to the granulosa layer (Hodges, 1974). The two layers are, however, physically separated by the basement membrane. As stated previously, small follicles are responsible for the majority of estrogen produced in the ovary. This estrogen is produced solely by the theca layer, as the granulosa cells of

small follicles are steroidogenically incompetent (Davidson *et al.*, 1979). The inability of granulosa cells from small follicles to produce steroids is due to the inactivity of the 17α -hydroxylase and C17,20 lyase enzymes (Tilly *et al.*, 1991). When the follicle reaches 2 - 4 mm in diameter, the theca layer differentiates into theca interna and theca externa cells (Nitta *et al.*, 1991). As the follicle approaches ovulation, enzyme activity (aromatase and 3β -hydroxy- Δ^5 -steroid dehydrogenase) in the theca layer decreases, culminating in the complete cessation of steroidogenic capacity in the theca cells of F₁ follicles (Huang *et al.*, 1979; Armstrong, 1982). It is not until the follicle reaches 9 - 12 mm in diameter that its granulosa cells are capable of producing progesterone. Furthermore, although LH receptors are present on smaller follicles, granulosa cells will not respond to LH until the follicle is 12 - 15 mm in diameter (Tilly *et al.*, 1991).

Currently, there are two theories that have been proposed to describe the steroidogenic events that take place in the avian ovary. Huang *et al.* (1979) proposed a two-cell model of follicular steroidogenesis which assumes that the theca layer is uniform in its steroidogenic function. This theory proposes that the majority of progestin production, as well as a small amount of androgen synthesis, is derived from the granulosa cells of pre-

ovulatory follicles. Progestins are secreted by granulosa cells and then further metabolized to androgens and estrogens in theca cells (Huang *et al.*, 1979). The more recent, three-cell theory of avian steroidogenesis proposes that progesterone produced in granulosa cells is metabolized by two distinct theca cell types (Porter *et al.*, 1989). In this model, theca interna cells utilize progesterone derived from granulosa cells to produce androgens, which are then passed to theca externa and converted to estrogens (Porter *et al.*, 1989). There has been very little research directed at the role of cytokines on reproduction in avians. Bryan *et al.* (1997) reported that human recombinant TNF α at a dose of 5 ng/ml consistently inhibited *in vitro* progesterone production among granulosa cells in the F₁, F₂, and F₃ (3 largest) follicles of the hen.

Advances in the field of genetics have given researchers new tools to aid in the study of reproductive function in the avian. Gross (1979) and Siegel and Gross (1980) developed two genetic lines of White Leghorn chickens selected for their ability to respond to an immune challenge after an injection of sheep red blood cells (SRBC). Birds in which high levels of antibodies were persistent five days after SRBC injection were termed high antibody (HA), while those with low levels were called low antibody (LA).

Using these two distinct lines, research has been conducted to determine whether the degree of antibody production effects the ability of a bird to resist bacterial and viral challenges. Both Hall *et al.* (1978) and Gross *et al.* (1980) found that the LA line exhibited a greater susceptibility to infection by *Mycoplasma gallisepticum*, coccidiosis, splenomegaly virus, New Castle Disease, and the northern fowl mite than did the HA line. Susceptibility to *Escherichia coli* and *Staphylococcus aureus* infection, however, was significantly greater in the HA line (Dunnington *et al.*, 1991 and Gross *et al.*, 1980). Gross *et al.* (1980) reported a 58% incidence of *E. coli* in the HA line compared to 35% for the LA line after inoculation of the posterior air sac with 10^{-3} to 10^{-4} cells. Similar results were reported in mice selected for low and high antibody response to SRBC (Biozzi *et al.*, 1979). These results suggest that strong antibody response is negatively correlated with the ability to defend against bacterial infection. The mechanisms by which birds defend themselves against invasion and infection may determine degree of resistance or susceptibility to specific diseases.

Differences in egg production characteristics between the LA and HA lines have also been reported. Siegel *et al.* (1982) found that hen-day egg production to 300 days was significantly lower in the HA line as compared

to the LA line. LA birds began egg production at an earlier age and at lower body weights than did HA birds (Siegel *et al.*, 1982 and Martin *et al.*, 1990). Martin *et al.* (1990) reported higher body weights for LA birds as measured at 4 and 24 weeks of age. Fertility was also different between the two lines with HA birds exhibiting reduced fertility at all ages (Seigel *et al.*, 1982).

The differences in weight gain, egg production, age of sexual maturity, and response to an immune challenge in these two lines imply that immuno-competence and reproductive function may somehow be related. The objectives of the following study were to: 1) determine the differences in plasma levels of P_4 and LH between HA and LA hens, 2) determine the effect of human versus murine $TNF\alpha$ on granulosa cell P_4 and P_5 secretion among HA and LA lines of hens, and 3) determine the differences in P_4 and P_5 production by granulosa cells during the ovulatory cycle as affected by human and murine $TNF\alpha$ in the presence of an ovulatory amount of ovine LH. By studying these parameters, it may be possible to gain a better understanding of the relationship between immune status and reproductive function in the domestic laying hen (*Gallus domesticus*).

2. MATERIALS AND METHODS

One hundred and twenty Single Comb White Leghorn hens selected for a high (HA) or low antibody (LA) response to immuno-challenge with sheep red blood cells (SRBC) were housed in individual cages at Cherokee Poultry Farm, University of Tennessee at Knoxville. Hens were allowed ad libitum access to feed and water on a lighting schedule consisting of 16 hours of light and 8 hours of darkness. Hens were fed a standard commercial poultry layer mash which contained 16% protein (Tennessee Farmers CO-OP, Laverne, TN). Egg records were maintained from hatch to 442 days of age and were updated daily in order to determine hen-day egg production (HDEP). Percent HDEP was defined as the percentage of eggs laid (egg production) in a given time period per number of live hens during that same period (hen-days; North, 1978). For example, if 4 hens produced 9 eggs in 3 days and none died, percent HDEP for the hens in those 3 days was 75% ($9 \text{ eggs} / (4 \text{ hens})(3 \text{ days}) \times 100 = 75$). Data collection began when flock HDEP reached 15% for each line. Experiment 1 was conducted to determine *in vivo* levels of circulating steroids in HA and LA hens during one ovulatory cycle. A second experiment was conducted to

determine *in vitro* steroid production of granulosa cells from HA and LA hens as affected by TNF α .

Experiment 1

At 14 and 34 weeks of egg production, hens were monitored for time of oviposition and serially bled via brachial vein cannulation as described by Johnson (1981) at 4, 8, 12, and 16 hours after oviposition, and subsequently every hour until the next oviposition. This timeline was reversed in order to reflect hours before oviposition. Three mls of blood was collected from each hen and centrifuged at 400 X g for 10 minutes in order to separate plasma from erythrocytes. Plasma was decanted into 12 x 75 mm borosilicate glass tubes (Fisher Scientific, Pittsburgh, PA) containing 50 ul saturated sodium citrate (Baxter, McGraw Park, IL) and stored at -20°C for later analysis. Hematocrits were determined every 3rd sample to ensure that packed cell volume did not drop more than 20% below baseline, which was determined at the time the cannula was seated. Erythrocytes were kept separate by hen, pooled, and combined with an equal volume of 0.9% saline and 50,000 U penicillin G (Sigma, St. Louis, MO). Erythrocytes were returned to the hen after every 3rd blood sample to maintain blood volume. Plasma was analyzed for progesterone (P4) using

a validated (Bryan *et. al.*, 1997) commercially available radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). Luteinizing hormone (LH) was measured by radioimmunoassay using specific antisera to chicken LH (USDA-AcLH-5), reference standards (USDA-cLH-K-3), and immuno-chemical grade hormone for radioiodination (USDA-cLH-I-3) according to the procedure of Johnson and van Tienhoven (1980).

Experiment 2

At 16 and 37 weeks of egg production, 5 birds from each line were sacrificed at 4, 12, 18, 24 hours post oviposition of a mid sequence egg. The ovary was removed from each hen, washed in Hank's basal salt solution (Gibco, Grand Island, N.Y.), 70% ethyl alcohol, and again in Hank's before storage in RPMI-1640 on ice. Ovaries were blotted dry and weighed. Follicles were measured (mm diameter), counted, and sorted by size (F_1 , F_2 , F_3 , 10-12 mm, 7-9 mm, 4-6 mm, and 1-3 mm). Atretic follicles, determined as those deflated or in a hemorrhaged state (Tilly *et. al.* 1991), were also counted. Upon removal of connective tissue and vasculature surrounding the three largest follicles (F_1 , F_2 , and F_3), the granulosa layer was microdissected from the theca layer using aseptic techniques. Granulosa layers from the three largest follicles of each hen

were pooled according to size, and individual cells isolated via digestion in a 2.5 mg/ml trypsin (Sigma, St. Louis , MO)/Hank's basal salt solution under constant agitation for 3 minutes at 37°C. Granulosa cells were centrifuged at 400 X g for 4 minutes and washed twice with RPMI-1640 (Gibco, Grand Island, NY). Cells were counted by trypan blue dye exclusion and plated at a concentration of 500,000 cells/0.5 ml in RPMI-1640 media, antibiotics (50 U penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin), and 0.1 % bovine serum albumin (Sigma, St. Louis, MO; hereafter called complete media). Treatments consisted of; 1) untreated media (control), 2) 5 ng/ml human recombinant TNF α (hTNF α ; R&D Systems, Minneapolis, MN), 3) 5 ng/ml murine recombinant TNF α (mTNF α ; R&D Systems), 4) 100 ng/ml ovine LH (oLH; National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Bethesda, MD), 5) hTNF α + oLH, or 6) mTNF α + oLH. Cells and their respective treatments were cultured for 3 hours in 95% air and 5% CO₂ at 39.5 °C. After incubation, cells were pelleted, the supernatant removed and stored in 12 x 75 borosilicate glass tubes at -20 °C for later analysis. Progesterone (P₄) secretion by granulosa cells was measured using the commercial RIA described previously (Bryan *et al.*, 1997). Pregnenolone (P₅) secretion by

granulosa cells was measured via radioimmunoassay and validated using antisera to pregnenolone-3 hemisuccinate (Accurate Chemical and Scientific Corp., Westbury, NY) and radio labeled 7-³H(N) pregnenolone (21.0 Ci/mmol; New England Nuclear, Boston, MA). The antiserum is known to cross-react with 17-hydroxypregnenolone (1.0%), progesterone (3.0%), 17-hydroxy-progesterone (1.4%), corticosterone (<0.4%), 11-deoxycorticosterone (<0.3%), cortisol (<0.3%), 11-deoxycortisol (<0.3%), 5 β -pregnan-3,20 dione (<0.4%), 5 α -pregnan-3,20 dione (4.5%), 5 α -pregnan-3 α 1,20 one (5.6%), 5 α -pregnan-3 α ,20 α diol (<0.45%), and 5 α -pregnan-3 β ,20 diol (<0.4%; Accurate Scientific and Chemical Corp., Westbury, NY). Reagents were diluted in 0.05 M sodium phosphate buffer containing 0.1% gelatin and 0.2% sodium azide, pH 7.4 (hereafter call reaction buffer). Into 12 x 75 mm glass borosilicate tubes were added 400 μ l reaction buffer, 100 μ l antiserum (1:23,000 dilution), 100 μ l ³H-P₅ (10,000 cpm), and 100 μ l standard or unknown. Standards (15.625 pg/ml - 4.0 ng/ml) were made by adding specific quantities of 5-pregnen-3 β -ol-20-one (Steraloids Inc., Newport, RI) to reaction buffer. Reaction mixtures were incubated for 30 minutes at 37°C, and then for 16 hours at 4°C. After incubation, 500 μ ls of 1:10 Dextran-T70/Norit A (Fisher Scientific,

Pittsburgh, PA) charcoal solution was added to each sample tube. Samples were held at 4°C for 10 minutes, and then centrifuged at 1,500 X g and 4°C (Beckman TJ-6; Fullerton, CA) for 15 minutes. Supernatants were decanted into 50 x 16 mm scintillation vials (Sarstedt, Newton, NC), mixed with 5 mls UltimaGold™ scintillation fluid (Packard, Downers Grove, IL), and counted in a liquid scintillation counter (Beckman LS5801, Fullerton, CA). Tests for parallelism were conducted using 35, 45, and 55 ul of media pooled from the 3 largest preovulatory follicles (Table 1). The optimal dilution of pregnenolone antiserum was determined to be 1/23,000 which agreed with the dilution suggested by the manufacturer (Table 2). Intra- and Inter-assay coefficients of variation for the assays were 5.2 and 11.6%, and 7.7 and 8.9% for P₄ and P₅, respectively.

Data Analysis

Data were analyzed using the Mixed procedure of the statistical analysis system (SAS, 1996). Data were log transformed prior to analysis to determine significance. Means were separated by Fisher's least significant difference test. Table values are reported as the least square (LS) mean of the non-log transformed data with significance denoted at $P \leq 0.05$.

Table 1. Test for Parallelism of Pregnenolone Radio-Immunoassay

Sample	Volume Assayed (μ l)	Pregnenolone* (ng/ml)
Pooled Media	35	13.99 $\pm$ 0.42
(F ₁ -F ₃ Follicles)	45	13.63 $\pm$ 0.33
	55	13.46 $\pm$ 1.07

*Mean $\pm$ standard error.

Table 2. Tests for Binding Efficiency of Pregnenolone Antiserum

Antibody Dilution*	Binding (%)
1:18,000	50.01
1:20,000	48.29
1:23,000	45.50
1:25,000	42.83
1:28,000	38.06

*Final dilution according to the manufacturer's instructions (Accurate Scientific and Chemical Corp., Westbury, NY).

3. RESULTS

Production Characteristics

Hens from the LA line reached puberty (first egg) and 15% HDEP earlier than did hens from the HA line (150 and 163 days of age versus 158 and 172 days of age, respectively). Because of this difference in reproductive age, LA hens were killed approximately 9 days prior to HA hens during *in vitro* experiments in order to maintain differences due to physiological age. Overall percent HDEP between the HA and LA lines (66.62 and 63.47%, respectively) did not differ significantly ($P>0.05$). However, if percent HDEP was measured from the day of first egg (day 150) to 442 days of age (cessation of the experiment), LA hens laid at a higher rate ($P\leq 0.05$), 64.53 versus 58.82%, respectively.

At both 16 and 37 weeks of egg production, ovary weight from HA hens was greater ($P\leq 0.05$) than that of LA hens (Table 3). In younger hens, the size of F_1 and F_3 follicles was not different between lines ($P>0.05$). There was also no difference in either the number of small, slowly growing (1-12 mm) or atretic follicles among hens from either genetic line (Table 4).

Table 3. Ovary weight and size of F₁, F₂, and F₃ follicles for HA and LA hens at 16 and 37 weeks of egg production*.

Follicle Weight, Size, or Number	Weeks of Egg Production			
	16		37	
	Genetic Line/(number of hens)		Genetic Line/(number of hens)	
	HA/(n=20)	LA/(n=20)	HA/(n=20)	LA/(n=20)
Ovary Weight (grams)	42.49 ± 1.60 ^{B†}	35.94 ± 1.56 ^C	47.80 ± 1.94 ^A	38.08 ± 1.60 ^{BC}
F ₁ (mm [‡])	33.58 ± 0.33 ^B	32.90 ± 0.33 ^B	35.19 ± 0.35 ^A	33.71 ± 0.33 ^B
F ₂ (mm)	29.97 ± 0.43 ^B	28.50 ± 0.40 ^C	32.03 ± 0.45 ^A	29.76 ± 0.41 ^B
F ₃ (mm)	25.47 ± 0.61 ^{AB}	24.15 ± 0.58 ^{BC}	26.47 ± 0.63 ^A	23.18 ± 0.60 ^C

*Values are reported as least square means ± standard error of the mean.

†^{A,B,C} Values in the same row with different letters are significantly different (P ≤ 0.05).

‡Millimeters diameter.

Table 4. Numbers of 10 - 12 mm, 7 - 9 mm, 4 - 6 mm, 1 - 3 mm, and atretic follicles for HA and LA hens at 16 and 37 weeks of egg production*.

Number of Follicles	Weeks of Egg Production			
	16		37	
	Genetic Line/(number of hens)		Genetic Line/(number of hens)	
	HA/(n=20)	LA/(n=20)	HA/(n=20)	LA/(n=20)
10 - 12 mm (No. [†])	1.21 ± 0.17 ^{A‡}	1.00 ± 0.17 ^A	1.06 ± 0.18 ^A	0.88 ± 0.17 ^A
7 - 9 mm (No.)	4.00 ± 0.67 ^A	5.30 ± 0.66 ^A	5.88 ± 0.71 ^A	5.84 ± 0.67 ^A
4 - 6 mm (No.)	13.00 ± 1.07 ^{AB}	11.75 ± 1.04 ^B	15.35 ± 1.13 ^A	11.16 ± 1.07 ^B
1 - 3 mm (No.)	26.32 ± 2.19 ^B	25.80 ± 2.13 ^B	38.06 ± 2.31 ^A	28.47 ± 2.10 ^B
Atretic Follicles	4.32 ± 0.56 ^{AB}	4.05 ± 0.54 ^B	5.71 ± 0.59 ^A	5.16 ± 0.56 ^{AB}

*Values are reported as least square means ± standard error of the mean.

† Number of Follicles

‡^{A,B,C} Values in the same row with different letters are significantly different (P ≤ 0.05).

At 37 weeks of egg production, all pre-ovulatory follicles (F_{1-3}) were larger ($P \leq 0.05$) among HA hens as compared to LA hens. Similarly, there were more ($P \leq 0.05$) 1-3 and 4-6 mm small white follicles among older HA hens, but not of 7-9 mm, 10-12 mm, or atretic follicles between lines.

In Vivo Steroid Secretion

Both plasma progesterone and luteinizing hormone were significantly higher ($P \leq 0.05$) among HA versus LA hens during the peak pre-ovulatory period of the ovulatory cycle (Figures 1 and 2). Specifically, circulating levels of progesterone (Figure 1) and luteinizing hormone (Figure 2) were greater ($P \leq 0.05$) among HA hens at 6, 5, 4, 3, 2, and 1 hours prior to oviposition (Figure 1).

In Vitro Steroid Secretion

Similar to plasma levels, *in vitro* progesterone production was higher ($P \leq 0.05$) among HA hens as compared to LA hens. This was apparent in all follicle sizes at both 16 and 37 weeks of egg production (Table 5). Progesterone production by granulosa cells from both genetic lines was less ($P \leq 0.05$) at 37 weeks of egg production, as compared to 16

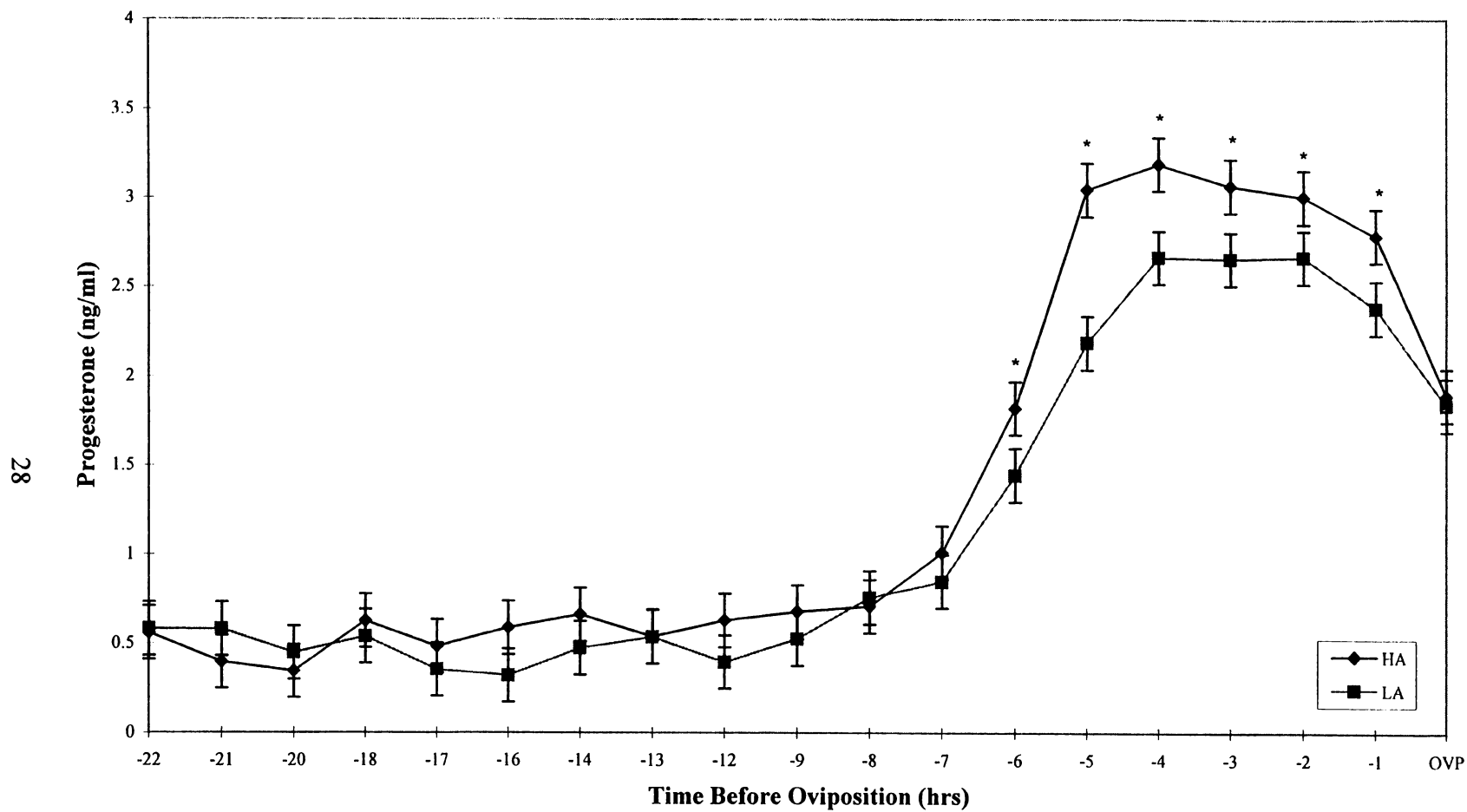


**Figure 1. Plasma progesterone (ng/ml) during one ovulatory cycle in HA and LA hens.
(combined data from samples collected at 14 and 35 weeks of egg production).**

*Indicates significant difference ($P \leq 0.05$).

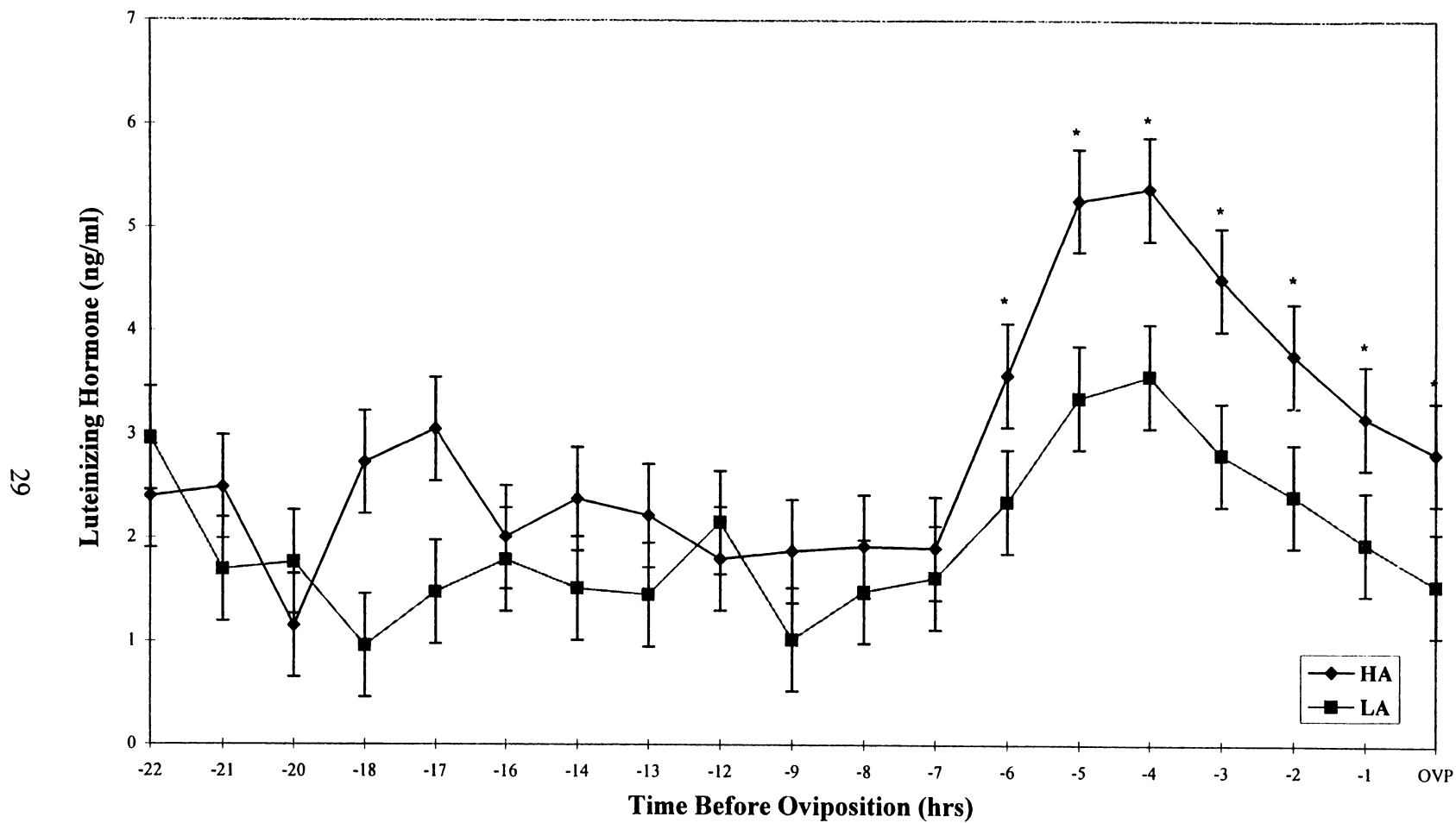


Figure 2. Plasma luteinizing hormone (ng/ml) during one ovulatory cycle in HA and LA hens (combined data from samples collected at 14 and 35 weeks of egg production).

*Indicates significant difference ($P \leq 0.05$).

Table 5. *In vitro* progesterone and pregnenolone (P4 and P5; ng/ml) secretion from granulosa cells of the 3 largest preovulatory follicles (F₁, F₂, and F₃) from HA and LA hens at 16 and 37 weeks of egg production.*

Steroid/Follicle Size	Weeks of Egg Production			
	16		37	
	Genetic Line/(number of hens)		Genetic Line/(number of hens)	
	HA/(n=20)	LA/(n=20)	HA/(n=20)	LA/(n=20)
P₄ (ng/ml)/				
F₁ follicle	242.35 ± 3.69 ^{A†a‡}	191.57 ± 3.35 ^{Ab}	189.89 ± 3.69 ^{Bc}	147.52 ± 3.41 ^{Bd}
F₂ follicle	148.25 ± 2.08 ^{Bc}	141.24 ± 2.13 ^{Bd}	271.75 ± 2.21 ^{Aa}	164.86 ± 2.13 ^{Ab}
F₃ follicle	129.86 ± 1.53 ^{Cb}	95.53 ± 1.55 ^{Cc}	152.62 ± 1.46 ^{Ca}	65.31 ± 1.53 ^{Cd}
P₅ (ng/ml)/				
F₁ follicle	12.70 ± 0.16 ^{Aab}	12.51 ± 0.14 ^{Aa}	12.24 ± 0.16 ^{Bb}	9.92 ± 0.14 ^{Ac}
F₂ follicle	10.35 ± 0.10 ^{Bb}	9.01 ± 0.10 ^{Bd}	15.02 ± 0.11 ^{Aa}	9.92 ± 0.10 ^{Ac}
F₃ follicle	8.24 ± 0.08 ^{Cb}	6.30 ± 0.08 ^{Cc}	10.90 ± 0.08 ^{Ca}	6.07 ± 0.08 ^{Bd}

*Values are reported as least square means ± standard error of the mean. Significant differences (P≤0.05) are denoted by letter differences between the means of log transformed data.

†^{A,B,C} Values within the same column and for the same steroid with different letters are significantly different (P≤0.05).

‡^{a,b,c,d} Values within the same row with different letters are significantly different (P≤0.05).

weeks of egg production. However, progesterone secretion was higher in F₂ follicles among both lines at 37 weeks of age ($P \leq 0.05$).

Similar differences were found in the production of pregnenolone by granulosa cells of HA and LA hens (Table 5). Pregnenolone secretion by granulosa cells was greater ($P \leq 0.05$) among HA hens in all follicle sizes and in both time periods with the exception that F₁ follicle secretion was the same at 16 weeks of egg production. As would be expected, pregnenolone production was less ($P \leq 0.05$) among the smaller pre-ovulatory follicles (F₂ and F₃) as compared to the F₁. However, as the hen aged, pregnenolone secretion was found to remain the same (F₁ follicle in HA and LA hens; $P > 0.05$) or increase (F₂ and F₃ follicles in LA hens; $P \leq 0.05$).

Steroid Secretion as Affected by TNF α

In vitro treatment of granulosa cells with recombinant TNF α had little effect on basal progesterone secretion. Treatment of F₁ and F₃ follicles with hTNF α , but not mTNF α , resulted in a reduction ($P \leq 0.05$) in progesterone secretion (Table 6). This decrease was evident in F₁ follicles among HA hens at 16 weeks of egg production and LA hens at 37 weeks of egg production. Similarly, progesterone production was reduced ($P \leq 0.05$)

Table 6. *In vitro* progesterone (ng/ml) production from granulosa cells of the F₁, F₂, and F₃ follicles in HA and LA hens at 16 and 37 weeks of egg production in response to various treatments.

Follicle Size/Treatment	Weeks of Egg Production			
	16		37	
	Genetic Line/(number of hens)		Genetic Line/(number of hens)	
	HA/(n=20)	LA/(n=20)	HA/(n=20)	LA/(n=20)
F₁ follicle/				
Control (nothing)	123.65 ^{C*a†}	90.46 ^{Db}	59.89 ^{Dc}	56.05 ^{Cc}
hTNF (5 ng/ml)	107.31 ^{Da}	96.54 ^{CDa}	61.58 ^{Db}	46.77 ^{Dc}
mTNF (5 ng/ml)	121.78 ^{Ca}	100.51 ^{Cb}	60.61 ^{Dc}	46.73 ^{Dd}
oLH (100 ng/ml)	330.57 ^{Ba}	273.33 ^{Bb}	311.03 ^{BCb}	217.68 ^{Bc}
oLH + hTNF	325.88 ^{Ba}	284.62 ^{ABb}	293.30 ^{Cb}	224.34 ^{Bc}
oLH + mTNF	444.89 ^{Aa}	303.95 ^{Ab}	352.91 ^{Ab}	293.59 ^{Ab}
Pooled SEM [‡]	3.69	3.35	3.69	3.41
F₂ follicle/				
Control (nothing)	91.70 ^{Cb}	80.55 ^{Cc}	110.34 ^{Da}	79.12 ^{Dc}
hTNF (5 ng/ml)	89.93 ^{Cb}	78.81 ^{Cc}	104.83 ^{Da}	82.63 ^{CDb}
mTNF (5 ng/ml)	92.30 ^{Cb}	81.24 ^{Cc}	117.75 ^{Da}	84.96 ^{Cbc}
oLH (100 ng/ml)	188.82 ^{Bc}	206.65 ^{ABc}	378.78 ^{Ca}	238.29 ^{Bb}
oLH + hTNF	194.33 ^{Bc}	192.99 ^{Bc}	431.72 ^{Ba}	235.48 ^{Bb}
oLH + mTNF	232.42 ^{Ac}	207.99 ^{Ad}	487.10 ^{Aa}	268.71 ^{Ab}
Pooled SEM	2.08	2.13	2.21	2.13
F₃ follicle/				
Control (nothing)	81.62 ^{Ca}	59.83 ^{Bb}	58.63 ^{Cb}	35.99 ^{Bc}
hTNF (5 ng/ml)	80.31 ^{Ca}	55.58 ^{Cb}	56.64 ^{Cb}	33.22 ^{Cc}
mTNF (5 ng/ml)	82.19 ^{Ca}	54.84 ^{Cc}	59.73 ^{Cb}	33.76 ^{Cd}
oLH (100 ng/ml)	171.04 ^{Bb}	127.20 ^{Ac}	234.61 ^{Ba}	95.32 ^{Ad}
oLH + hTNF	174.87 ^{Bb}	140.93 ^{Ac}	232.35 ^{Ba}	96.33 ^{Ad}
oLH + mTNF	189.16 ^{Ab}	134.80 ^{Ac}	273.75 ^{Aa}	97.21 ^{Ad}
Pooled SEM	1.53	1.55	1.46	1.53

*A,B,C,D Values in the same column and for the same follicle size with different letters are significantly different ($P \leq 0.05$).

†^{a,b,c,d} Values in the same row with different letters are significantly different ($P \leq 0.05$).

‡Standard error of the mean.

in F₃ follicles among LA hens at both time periods. Human TNF α had no effect ($P>0.05$) on progesterone secretion from F₂ granulosa cells, regardless of genetic line or age.

Treatment of granulosa cells with oLH resulted in a large increase ($P\leq 0.05$) in progesterone secretion which was further increased ($P\leq 0.05$) after co-treatment with mTNF α (Table 6), however this effect was lost in F₃ LA follicles. Co-treatment of granulosa cells with hTNF α and oLH did not ($P>0.05$) result in an additive increase in progesterone secretion over that observed with oLH alone.

As observed with progesterone secretion, *in vitro* pregnenolone secretion declined in response to treatment with hTNF α , but not mTNF α (Table 7). This effect was more consistent among younger versus older hens, HA versus LA hens, and in larger follicles (F₁ vs. F₂ and F₃). Pregnenolone secretion was less ($P\leq 0.05$) after exposure to hTNF α in F₁ follicles among HA hens at 16 weeks of egg production, and LA hens at 37 weeks of egg production. Similarly, less ($P\leq 0.05$) pregnenolone was produced by F₂ granulosa cells from HA hens at both time periods, and F₃ granulosa cells from both lines at 16 weeks of egg production in response to treatment with hTNF α .

Table 7. *In vitro* pregnenolone (ng/ml) production from granulosa cells of the F₁, F₂, and F₃ follicles in HA and LA hens at 16 and 37 weeks of egg production in response to various treatments.

Follicle Size/Treatment	Weeks of Egg Production			
	16		37	
	Genetic Line/(number of hens)		Genetic Line/(number of hens)	
	HA/(n=20)	LA/(n=20)	HA/(n=20)	LA/(n=20)
F₁ follicle/				
Control (nothing)	7.32 ^{C*a†}	6.65 ^{Cb}	6.22 ^{CDb}	5.48 ^{Cc}
hTNF (5 ng/ml)	6.87 ^{Da}	6.60 ^{Ca}	5.89 ^{Db}	4.53 ^{Dc}
mTNF (5 ng/ml)	6.94 ^{Da}	6.89 ^{Ca}	6.40 ^{Ca}	5.15 ^{Cb}
oLH (100 ng/ml)	17.11 ^{Bb}	17.23 ^{Bab}	18.30 ^{Aba}	13.73 ^{Bc}
oLH + hTNF	17.75 ^{Ba}	18.14 ^{Aba}	17.52 ^{Ba}	14.03 ^{Bb}
oLH + mTNF	20.14 ^{Aa}	19.54 ^{Aa}	19.13 ^{Aa}	16.60 ^{Ab}
Pooled SEM [‡]	0.16	0.14	0.16	0.14
F₂ follicle/				
Control (nothing)	7.14 ^{Cb}	5.79 ^{Cd}	7.95 ^{Da}	6.34 ^{Cc}
hTNF (5 ng/ml)	6.67 ^{Db}	5.50 ^{Cd}	7.42 ^{Ea}	6.16 ^{Cc}
mTNF (5 ng/ml)	7.00 ^{CDb}	5.55 ^{Cd}	7.96 ^{Da}	6.19 ^{Cc}
oLH (100 ng/ml)	13.27 ^{Bb}	12.09 ^{Bc}	20.79 ^{Ca}	13.47 ^{Bb}
oLH + hTNF	13.60 ^{ABb}	12.13 ^{Bc}	21.93 ^{Ba}	13.21 ^{Bb}
oLH + mTNF	14.45 ^{Ab}	12.98 ^{Ac}	24.07 ^{Aa}	14.16 ^{Ab}
Pooled SEM	0.10	0.10	0.11	0.10
F₃ follicle/				
Control (nothing)	5.94 ^{Ca}	4.80 ^{Bb}	5.99 ^{Ca}	4.11 ^{Bc}
hTNF (5 ng/ml)	5.23 ^{Db}	4.49 ^{Cc}	5.72 ^{Ca}	4.05 ^{Bd}
mTNF (5 ng/ml)	5.78 ^{Ca}	4.89 ^{Bb}	5.79 ^{Ca}	4.17 ^{Bc}
oLH (100 ng/ml)	10.80 ^{ABb}	7.75 ^{Ac}	15.18 ^{Ba}	8.15 ^{Ac}
oLH + hTNF	10.60 ^{Bb}	8.07 ^{Ac}	16.10 ^{Aa}	7.81 ^{Ac}
oLH + mTNF	11.06 ^{Ab}	7.78 ^{Ac}	16.61 ^{Aa}	8.12 ^{Ac}
Pooled SEM	0.08	0.08	0.08	0.08

*A,B,C,D Values in the same column and for the same follicle size with different letters are significantly different (P≤0.05).

†^{a,b,c,d} Values in the same row with different letters are significantly different (P≤0.05).

‡Standard error of the mean.

Similar to the effect on progesterone secretion, treatment of granulosa cells with oLH resulted in a large increase ($P \leq 0.05$) in pregnenolone secretion (Table 7). Co-treatment of granulosa cells with mTNF α and oLH caused a further increase ($P \leq 0.05$) in pregnenolone secretion over that of oLH alone in all preovulatory follicles. This increase was predominant among F₁ and F₂ follicles, regardless of genetic line or age. F₃ follicles were basically unresponsive ($P > 0.05$) to co-treatment with oLH and either mouse or human TNF α . The significant increase ($P \leq 0.05$) in pregnenolone secretion due to oLH was not changed by addition of either form of the cytokine.

Steroid Secretion as Affected by TNF α During the Ovulatory Cycle

F1 follicles:

Analysis of data by time-point during the ovulatory cycle showed that the effect of TNF α on basal progesterone secretion of granulosa cells from young hens tended to occur very early (4 hrs) or very late (24 hrs) after oviposition of the previous egg (Table 8). The decline ($P \leq 0.05$) in progesterone secretion evident in HA hens (as described in Table 6) was due to a reduction at 4 and 24 hours after oviposition. LA hens appeared to

Table 8. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of the F₁ follicle at 4, 12, 18, and 24 hours after oviposition in HA and LA hens at 16 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	138.10 ^{C*a†}	147.03 ^{Ca}	161.94 ^{Ca}	47.55 ^{Be}	71.72 ^{Bd}	97.93 ^{Bbc}	108.99 ^{Cb}	83.17 ^{Bcd}
hTNF (5 ng/ml)	114.63 ^{Db}	135.39 ^{Ca}	143.53 ^{Ca}	35.69 ^{Cd}	81.39 ^{Bc}	89.85 ^{Bc}	126.84 ^{BCab}	88.07 ^{Bc}
mTNF (5 ng/ml)	134.02 ^{CDb}	142.52 ^{Cab}	166.73 ^{Ca}	43.83 ^{Bc}	75.80 ^{Bd}	88.37 ^{Bc}	146.17 ^{Bab}	91.69 ^{Bc}
oLH (100 ng/ml)	327.00 ^{Bc}	315.35 ^{Bc}	564.96 ^{Ba}	114.98 ^{Ae}	135.79 ^{Ae}	303.89 ^{Ac}	444.22 ^{Ab}	209.42 ^{Ad}
oLH + hTNF	318.33 ^{Bb}	361.90 ^{Bb}	506.78 ^{Ba}	116.50 ^{Ad}	136.80 ^{Ad}	334.51 ^{Ab}	442.85 ^{Aa}	224.31 ^{Ac}
oLH + mTNF	458.65 ^{Ab}	491.12 ^{Ab}	693.32 ^{Aa}	136.47 ^{Ae}	148.71 ^{Ae}	330.86 ^{Ac}	495.34 ^{Ab}	240.90 ^{Ad}
Pooled SEM [‡]	6.86	7.02	6.70	8.73	6.70	6.86	6.53	6.70
Pregnenolone (ng/ml)/								
Control (nothing)	6.90 ^{Cc}	6.64 ^{Cc}	11.34 ^{Ca}	4.65 ^{Ce}	5.32 ^{Cd}	5.25 ^{Bd}	6.99 ^{Cc}	9.04 ^{Bb}
hTNF (5 ng/ml)	6.52 ^{CDcd}	6.22 ^{Cd}	10.89 ^{Ca}	3.84 ^{Cf}	6.00 ^{BCd}	5.19 ^{Be}	7.31 ^{Cbc}	7.91 ^{Cb}
mTNF (5ng/ml)	5.95 ^{Dd}	5.99 ^{Cd}	11.60 ^{Ca}	4.22 ^{Cf}	6.13 ^{Bcd}	4.89 ^{Be}	7.19 ^{Cc}	9.34 ^{Bb}
oLH (100ng/ml)	14.33 ^{Bc}	13.50 ^{Bc}	31.42 ^{Ba}	9.19 ^{Bd}	11.11 ^{Ad}	13.35 ^{Ac}	22.69 ^{Bb}	21.76 ^{Ab}
oLH + hTNF	15.28 ^{ABc}	13.75 ^{Bcd}	31.11 ^{Ba}	10.86 ^{Ae}	11.84 ^{Ade}	14.09 ^{Ac}	21.89 ^{Bb}	24.73 ^{Ab}
oLH + mTNF	16.93 ^{Ac}	16.40 ^{Ac}	36.62 ^{Aa}	10.62 ^{Ad}	11.40 ^{Ad}	15.07 ^{Ac}	27.20 ^{Ab}	24.50 ^{Ab}
Pooled SEM	0.29	0.30	0.29	0.38	0.29	0.29	0.29	0.29

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different ($P \leq 0.05$).

†^{a,b,...,f} Values in the same row with different letters are significantly different ($P \leq 0.05$).

‡Standard Error of the Mean.

be unresponsive to either form of $\text{TNF}\alpha$. The one exception was that m $\text{TNF}\alpha$ caused an increase ($P \leq 0.05$) in progesterone secretion 18 hours after oviposition.

The increased ($P \leq 0.05$) progesterone production after co-treatment of F_1 granulosa cells with m $\text{TNF}\alpha$ and oLH was found to occur 4, 12, and 18 hours after oviposition in young HA hens (Table 8). LA hens were found to be unresponsive to co-treatment with oLH and m $\text{TNF}\alpha$. Ovine LH induced progesterone secretion from F_1 follicles of younger hens from each genetic line was not affected by co-treatment with h $\text{TNF}\alpha$.

Analysis of pregnenolone data obtained during the ovulatory cycle showed that the h $\text{TNF}\alpha$ induced decrease ($P \leq 0.05$) occurred 4 hours after oviposition in HA hens, and 4 and 24 hours after oviposition in LA hens (Table 8).

The addition of m $\text{TNF}\alpha$ (but not h $\text{TNF}\alpha$) plus oLH consistently stimulated the production of pregnenolone by F_1 granulosa cells over that of oLH alone, throughout the ovulatory cycle in younger hens from both lines (Table 8). This was significant in HA hens at all times during the ovulatory cycle, and in LA hens 18 hours after oviposition.

Among older hens, the time response to $\text{TNF}\alpha$ was not observed in either genetic line with few exceptions (Table 9). Progesterone secretion increased ($P \leq 0.05$) after exposure to h $\text{TNF}\alpha$ in HA hens 12 hours after oviposition. In LA hens, 24 hours after oviposition, both forms of $\text{TNF}\alpha$ reduced ($P \leq 0.05$) F_1 follicle progesterone secretion.

As in younger hens, granulosa cells from F_1 follicles of older hens co-treated with m $\text{TNF}\alpha$ (but not h $\text{TNF}\alpha$) and oLH consistently produced more ($P \leq 0.05$) progesterone throughout the ovulatory cycle than cells treated with oLH alone (Table 9). Co-treatment resulted in higher ($P \leq 0.05$) progesterone secretion at 4 and 18 hours after oviposition in HA hens, and 4, 12, and 24 hours after oviposition in LA hens.

Differences in pregnenolone production due to *in vitro* treatment of F_1 granulosa cells in older hens with h $\text{TNF}\alpha$ appeared to be dependent on genetic line (Table 9). While treatment with h $\text{TNF}\alpha$ suppressed ($P \leq 0.05$) pregnenolone production at all times during the ovulatory cycle in LA hens, no effect ($P > 0.05$) was seen in HA hens, regardless of time. As expected, mouse $\text{TNF}\alpha$ had no effect ($P > 0.05$) on the secretion of pregnenolone at any time during the ovulatory cycle in either genetic line.

Table 9. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of the F₁ follicle at 4, 12, 18, and 24 hours after oviposition in HA and LA hens at 37 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	52.39 ^{C*bc†}	48.80 ^{Dc}	63.26 ^{Cab}	75.12 ^{Ba}	37.91 ^{Cd}	60.38 ^{Cb}	56.33 ^{Bbc}	69.58 ^{Cab}
hTNF (5 ng/ml)	54.22 ^{Cb}	57.99 ^{Cb}	60.53 ^{Cab}	73.58 ^{Ba}	38.44 ^{Cc}	52.85 ^{Cbc}	50.49 ^{Bbc}	45.29 ^{Dc}
mTNF (5 ng/ml)	50.01 ^{Cbc}	61.29 ^{Cab}	58.11 ^{Cb}	73.01 ^{Ba}	37.09 ^{Cd}	53.26 ^{Cbc}	49.67 ^{Bbc}	46.88 ^{Dc}
oLH (100 ng/ml)	128.94 ^{Be}	638.54 ^{ABa}	167.06 ^{Bd}	309.57 ^{Ab}	232.45 ^{Bc}	300.75 ^{Bb}	157.77 ^{Ad}	179.74 ^{Bd}
oLH + hTNF	139.19 ^{ABd}	586.28 ^{Ba}	182.51 ^{Bc}	265.23 ^{Ab}	257.68 ^{Bb}	279.08 ^{Bb}	149.55 ^{Ad}	211.04 ^{Bc}
oLH + mTNF	152.68 ^{Ae}	735.94 ^{Aa}	231.06 ^{Ad}	291.97 ^{Ac}	329.45 ^{Abc}	372.27 ^{Ab}	141.90 ^{Ae}	330.28 ^{Abc}
Pooled SEM [‡]	6.70	6.53	7.02	8.98	6.53	6.70	6.70	7.34
Pregnenolone (ng/ml)/								
Control (nothing)	6.21 ^{Ba}	6.10 ^{Cab}	6.36 ^{Cab}	6.23 ^{Cab}	4.57 ^{Cc}	5.44 ^{Bb}	6.12 ^{Bab}	5.78 ^{Cab}
hTNF (5ng/ml)	5.96 ^{Bab}	6.19 ^{Ca}	5.53 ^{Cab}	5.87 ^{Cab}	3.85 ^{Dc}	4.37 ^{Cc}	5.22 ^{Cb}	4.67 ^{Dbc}
mTNF (5ng/ml)	6.61 ^{Ba}	7.03 ^{Ba}	6.02 ^{Cc}	5.93 ^{Ccd}	4.86 ^{Cd}	5.04 ^{Bd}	5.60 ^{BCcd}	5.09 ^{CDd}
oLH (100ng/ml)	11.48 ^{Ac}	28.65 ^{Aa}	16.05 ^{ABb}	17.03 ^{ABb}	15.74 ^{Bb}	16.69 ^{Ab}	11.37 ^{Ac}	11.11 ^{Bc}
oLH + hTNF	11.98 ^{Ac}	27.74 ^{Aa}	14.91 ^{Bbc}	15.45 ^{Bb}	15.87 ^{Bb}	16.15 ^{Ab}	12.05 ^{Ac}	12.04 ^{Bc}
oLH + mTNF	12.58 ^{Ac}	28.65 ^{Aa}	16.84 ^{Ab}	18.47 ^{Ab}	18.63 ^{Ab}	18.07 ^{Ab}	11.88 ^{Ac}	17.82 ^{Ab}
Pooled SEM	0.29	0.28	0.29	0.38	0.29	0.27	0.29	0.30

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different ($P \leq 0.05$).

†^{a,b,...f} Values in the same row with different letters are significantly different ($P \leq 0.05$).

‡Standard error of the mean.

Pregnenolone production by F₁ granulosa cells was further increased ($P \leq 0.05$) over that of oLH alone in response to co-treatment with mTNF α in LA, but not HA, hens at 37 weeks of egg production (Table 9). Specifically, these cells secreted more ($P \leq 0.05$) pregnenolone 4 and 24 hours after oviposition.

F₂ follicles:

Basal production of progesterone by F₂ granulosa cells measured at 4, 12, 18, or 24 hours after oviposition was unaffected ($P > 0.05$) by treatment with either form of TNF α with one exception (Tables 10 and 11). As with F₁ follicles, F₂ progesterone secretion was less ($P \leq 0.05$) 4 hours after oviposition among older HA hens in response to hTNF α .

In vitro treatment of F₂ follicles with mTNF α and oLH consistently resulted in higher ($P \leq 0.05$) progesterone secretion as compared to treatment with oLH alone among hens of both genetic lines and at either age (Tables 10 and 11). This was most evident in HA versus LA hens (Table 10).

Basal pregnenolone secretion was only reduced ($P \leq 0.05$) after treatment with hTNF α 12 hours after oviposition in either genetic line at 16

Table 10. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of the F₂ follicle at 4, 12, 18, and 24 hours after oviposition in HA and LA hens at 16 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	97.84 ^{C*^b†}	100.17 ^{Cb}	131.20 ^{Ca}	37.59 ^{Ce}	49.78 ^{Cd}	95.77 ^{Bb}	117.71 ^{Ba}	58.93 ^{Bc}
hTNF (5 ng/ml)	98.26 ^{Cc}	100.49 ^{Cbc}	122.92 ^{Ca}	38.05 ^{Ce}	50.60 ^{Cd}	98.60 ^{Bbc}	114.20 ^{Bab}	51.83 ^{Bd}
mTNF (5 ng/ml)	100.22 ^{Cb}	101.30 ^{Cb}	129.41 ^{Ca}	38.26 ^{Cd}	52.69 ^{Cc}	91.62 ^{Bb}	126.64 ^{Ba}	54.01 ^{Bc}
oLH (100 ng/ml)	192.93 ^{Bc}	195.66 ^{Bc}	273.05 ^{Bb}	93.61 ^{Be}	123.37 ^{Bd}	186.59 ^{Ac}	400.53 ^{Aa}	116.08 ^{Ad}
oLH + hTNF	197.09 ^{Bc}	222.25 ^{ABbc}	253.74 ^{Bb}	104.26 ^{ABd}	112.23 ^{Bd}	203.09 ^{Ac}	343.14 ^{Aa}	110.42 ^{Ad}
oLH + mTNF	239.79 ^{Abc}	245.60 ^{Ab}	329.46 ^{Aa}	114.82 ^{Ac}	141.45 ^{Ad}	209.29 ^{Ac}	373.53 ^{Aa}	07.71 ^{Ac}
Pooled SEM [‡]	4.21	4.21	4.00	4.21	4.32	4.42	4.32	4.00
Pregnenolone (ng/ml)/								
Control (nothing)	6.90 ^{Cc}	6.97 ^{Bc}	9.71 ^{Ba}	4.99 ^{Be}	3.91 ^{Bf}	5.85 ^{CDd}	8.41 ^{Bb}	5.00 ^{Ce}
hTNF (5 ng/ml)	6.33 ^{Cc}	5.80 ^{Ccd}	9.65 ^{Ba}	4.91 ^{Bd}	4.29 ^{Be}	5.36 ^{Dd}	7.25 ^{Cb}	5.11 ^{Cd}
mTNF (5 ng/ml)	6.73 ^{Cbc}	6.51 ^{BCbc}	9.61 ^{Ba}	5.14 ^{Bd}	4.25 ^{Be}	6.07 ^{Cc}	7.25 ^{Cb}	4.63 ^{Cd}
oLH (100 ng/ml)	12.67 ^{Bcd}	11.76 ^{Ad}	19.48 ^{Aa}	9.16 ^{Ae}	9.99 ^{Ae}	13.64 ^{Bc}	16.36 ^{Ab}	8.37 ^{ABe}
oLH + hTNF	12.56 ^{Bc}	12.82 ^{Ac}	19.02 ^{Aa}	9.98 ^{Ad}	10.80 ^{Ad}	13.65 ^{Bc}	15.92 ^{Ab}	8.14 ^{Be}
oLH + mTNF	14.03 ^{Abc}	13.02 ^{Ac}	20.82 ^{Aa}	9.93 ^{Ade}	11.45 ^{Ad}	15.75 ^{Ab}	15.60 ^{Ab}	9.13 ^{Ae}
Pooled SEM	0.20	0.22	0.20	0.21	0.19	0.21	0.21	0.20

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different (P≤0.05).

†a,b,...f Values in the same row with different letters are significantly different (P≤0.05).

‡Standard error of the mean.

Table 11. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of the F₂ follicle at 4, 12, 18, and 24 hours after oviposition in HA and LA hens at 37 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	76.30 ^{C*cd†}	197.71 ^{Da}	98.03 ^{Cb}	69.35 ^{Bd}	40.00 ^{Ce}	79.85 ^{Cc}	104.51 ^{Bb}	92.13 ^{Bb}
hTNF (5 ng/ml)	65.08 ^{Dd}	187.56 ^{Da}	97.19 ^{Cb}	69.49 ^{Bd}	48.68 ^{Ce}	80.91 ^{Cc}	103.33 ^{Bb}	97.61 ^{Bb}
mTNF (5 ng/ml)	74.49 ^{CDd}	214.96 ^{Da}	106.91 ^{Cbc}	74.66 ^{Bd}	48.03 ^{Ce}	87.81 ^{Cc}	109.72 ^{Bb}	94.26 ^{Bc}
oLH (100 ng/ml)	226.99 ^{Bd}	679.54 ^{Ca}	179.05 ^{Be}	429.52 ^{Ab}	146.15 ^{Bf}	258.79 ^{Bd}	312.79 ^{Ac}	235.42 ^{Ad}
oLH + hTNF	256.29 ^{ABcd}	830.86 ^{Ba}	230.20 ^{Ad}	409.53 ^{Ab}	158.31 ^{Be}	279.30 ^{ABc}	277.68 ^{Ac}	226.64 ^{Ad}
oLH + mTNF	275.45 ^{Ac}	969.65 ^{Aa}	233.79 ^{Ae}	469.51 ^{Ab}	206.16 ^{Ae}	311.26 ^{Ac}	313.14 ^{Ac}	244.26 ^{Ad}
Pooled SEM [‡]	4.32	4.61	4.42	4.32	4.32	4.32	4.21	4.21
Pregnenolone (ng/ml)/								
Control (nothing)	5.85 ^{Be}	10.47 ^{Ba}	8.82 ^{Cb}	6.65 ^{Ccd}	4.43 ^{Bf}	6.45 ^{Cd}	7.40 ^{Bc}	7.07 ^{Bcd}
hTNF (5 ng/ml)	5.59 ^{Bc}	9.82 ^{Ba}	8.18 ^{Cb}	6.10 ^{Cc}	4.67 ^{Bd}	5.94 ^{Cc}	7.96 ^{Bb}	6.08 ^{Cc}
mTNF (5ng/ml)	6.10 ^{Bd}	10.78 ^{Ba}	8.46 ^{Cb}	6.52 ^{Cd}	4.38 ^{Be}	6.08 ^{Cd}	7.54 ^{Bbc}	6.77 ^{Bcd}
oLH (100 ng/ml)	17.07 ^{Ac}	31.02 ^{Aa}	14.10 ^{Bd}	20.99 ^{Bb}	11.72 ^{Ae}	13.21 ^{Bde}	15.87 ^{Ac}	13.06 ^{Ade}
oLH + hTNF	17.08 ^{Ac}	31.81 ^{Aa}	16.54 ^{Ac}	22.30 ^{Bb}	11.64 ^{Ae}	13.69 ^{Bde}	15.07 ^{Ad}	12.44 ^{Ae}
oLH + mTNF	17.05 ^{Ac}	34.65 ^{Aa}	18.24 ^{Ac}	26.34 ^{Ab}	11.78 ^{Af}	15.63 ^{Ad}	15.92 ^{Ad}	13.31 ^{Ae}
Pooled SEM	0.21	0.21	0.21	0.22	0.22	0.22	0.20	0.20

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different (P≤0.05).

†a,b,...f Values in the same row with different letters are significantly different (P≤0.05).

‡Standard error of the mean.

weeks of egg production (Tables 10 and 11). As before, mTNF α had no effect ($P>0.05$) on basal secretion of pregnenolone.

Co-treatment of F₂ granulosa cells with a combination of either mTNF α or hTNF α and oLH did not increase ($P>0.05$) F₂ follicle pregnenolone production from hens of either genetic line with few exceptions (Tables 10 and 11). Pregnenolone secretion was higher ($P\leq 0.05$) among younger HA hens 4 hours after oviposition, and 18 and 24 hours after oviposition in older HA hens after *in vitro* treatment with mTNF α and oLH. In LA hens of both ages, pregnenolone production increased ($P\leq 0.05$) 12 hours after oviposition in response to co-treatment with mTNF α plus LH.

F3 follicles:

Only isolated effects on F₃ follicle progesterone production were observed due to treatment with hTNF α (Tables 12 and 13). Reduced ($P\leq 0.05$) progesterone production was observed in HA hens 4 hours after oviposition at both 16 and 37 weeks of egg production. F₃ follicle progesterone secretion was less ($P\leq 0.05$) among LA hens 4 hours after oviposition at 16 weeks of egg production, and 18 hours after oviposition at both ages. Treatment of F₃ follicles from younger LA hens with mTNF α

Table 12. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of F₃ follicles at 4, 12, 18, and 24 hours after oviposition in HA and LA hens at 16 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	78.61 ^{C*c†}	86.34 ^{Cbc}	127.08 ^{Ba}	34.45 ^{CDc}	37.26 ^{Be}	56.48 ^{Cd}	92.98 ^{Cb}	52.58 ^{Bd}
hTNF (5 ng/ml)	67.45 ^{Dd}	93.64 ^{Cb}	123.07 ^{Ba}	37.08 ^{Cf}	31.88 ^{Cg}	59.84 ^{BCd}	78.30 ^{Dc}	52.30 ^{BCe}
mTNF (5 ng/ml)	72.20 ^{CDcd}	90.30 ^{Cb}	135.95 ^{Ba}	30.31 ^{Df}	30.99 ^{Cf}	64.39 ^{Bd}	77.62 ^{Dc}	46.36 ^{Ce}
oLH (100 ng/ml)	195.86 ^{Bb}	206.02 ^{Bb}	242.95 ^{Aa}	39.32 ^{Bf}	117.61 ^{Ad}	143.51 ^{Ac}	156.47 ^{Bc}	91.21 ^{Ae}
oLH + hTNF	218.37 ^{ABa}	207.72 ^{Ba}	230.66 ^{Aa}	42.74 ^{ABe}	121.27 ^{Ac}	148.44 ^{Ab}	204.18 ^{Aa}	89.80 ^{Ad}
oLH + mTNF	242.67 ^{Aa}	235.67 ^{Aa}	229.97 ^{Aa}	48.32 ^{Ae}	125.05 ^{Ac}	154.60 ^{Ab}	171.08 ^{Bb}	88.46 ^{Ad}
Pooled SEM [‡]	2.89	2.82	3.17	3.30	2.82	2.89	3.66	2.96
Pregnenolone (ng/ml)								
Control (nothing)	5.27 ^{Bc}	5.48 ^{Cbc}	8.60 ^{Ba}	4.39 ^{Cd}	3.86 ^{Be}	4.92 ^{Bcd}	5.97 ^{Cb}	4.45 ^{Bd}
hTNF (5ng/ml)	4.66 ^{Ccd}	4.84 ^{Dc}	7.96 ^{Ba}	3.47 ^{De}	3.23 ^{Ce}	4.23 ^{Cd}	6.40 ^{BCb}	4.10 ^{BCd}
mTNF (5 ng/ml)	5.52 ^{Bc}	5.27 ^{CDc}	8.54 ^{Ba}	3.80 ^{De}	4.29 ^{Bde}	4.62 ^{BCd}	6.68 ^{Bb}	3.98 ^{Ce}
oLH (100 ng/ml)	11.55 ^{Ab}	10.42 ^{Bbc}	16.00 ^{Aa}	5.25 ^{Ag}	7.11 ^{Be}	8.00 ^{Ad}	9.65 ^{Ac}	6.24 ^{Af}
oLH + hTNF	11.39 ^{Ab}	11.38 ^{ABb}	15.21 ^{Aa}	4.41 ^{Be}	8.07 ^{Ac}	7.78 ^{Ac}	10.42 ^{Ab}	6.01 ^{Ad}
oLH + mTNF	12.24 ^{Ab}	11.76 ^{Ab}	15.22 ^{Aa}	5.01 ^{Af}	6.92 ^{Bde}	7.36 ^{Ad}	10.42 ^{Ac}	6.41 ^{Ac}
Pooled SEM	0.15	0.16	0.16	0.19	0.15	0.15	0.19	0.15

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different ($P \leq 0.05$).

†a,b,...g Values in the same row with different letters are significantly different ($P \leq 0.05$).

‡Standard error of the mean.

Table 13. *In vitro* progesterone and pregnenolone production (ng/ml) by granulosa cells of F₃ follicles at 4, 12, 18 and 24 hours after oviposition in HA and LA hens at 37 weeks of egg production.

Steroid/Treatment	Genetic Line							
	HA				LA			
	Hours after Oviposition/(number of hens)				Hours after Oviposition/(number of hens)			
	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)	4/(n=5)	12/(n=5)	18/(n=5)	24/(n=5)
Progesterone (ng/ml)/								
Control (nothing)	54.72 ^{C*bc†}	71.20 ^{Da}	57.40 ^{Bc}	57.18 ^{Cbc}	12.50 ^{Be}	60.59 ^{Bb}	33.88 ^{Bd}	37.00 ^{BCd}
hTNF (5 ng/ml)	44.98 ^{Dc}	70.85 ^{Da}	54.28 ^{Bb}	56.45 ^{Cb}	11.44 ^{Bf}	53.95 ^{Bb}	28.23 ^{Ce}	39.27 ^{Bd}
mTNF (5 ng/ml)	48.37 ^{Dc}	80.24 ^{Ca}	57.24 ^{Bb}	53.05 ^{Cbc}	12.00 ^{Be}	56.85 ^{Bb}	32.72 ^{Bd}	33.46 ^{Cd}
oLH (100 ng/ml)	182.79 ^{Bb}	325.81 ^{Ba}	98.22 ^{Ad}	331.60 ^{Ba}	50.62 ^{Ae}	115.58 ^{Ac}	102.62 ^{Ac}	112.46 ^{Ac}
oLH + hTNF	162.18 ^{Bc}	300.25 ^{Bb}	99.73 ^{Ac}	362.25 ^{Ba}	54.33 ^{Af}	114.22 ^{Ad}	91.65 ^{Ac}	125.12 ^{Ad}
oLH + mTNF	210.78 ^{Ab}	366.86 ^{Aa}	107.47 ^{Ad}	409.90 ^{Aa}	51.98 ^{Af}	127.98 ^{Ac}	94.78 ^{Ae}	114.11 ^{Ac}
Pooled SEM [‡]	2.89	2.75	3.03	2.96	2.82	3.30	3.17	2.89
Pregnenolone (ng/ml)/								
Control (nothing)	5.40 ^{Cc}	6.86 ^{Ba}	5.68 ^{Cbc}	6.01 ^{Cb}	2.53 ^{CDe}	5.13 ^{Bc}	4.56 ^{Cd}	4.23 ^{Bd}
hTNF (5 ng/ml)	5.38 ^{Cb}	6.69 ^{Ba}	5.25 ^{Cb}	5.56 ^{Cb}	2.42 ^{Dd}	5.51 ^{Bb}	3.96 ^{Dc}	4.32 ^{Bc}
mTNF (5 ng/ml)	5.34 ^{Cbc}	7.44 ^{Ba}	5.51 ^{Cb}	4.87 ^{Dc}	2.78 ^{Cd}	5.39 ^{Bbc}	4.28 ^{CDc}	4.22 ^{Bc}
oLH (100 ng/ml)	12.40 ^{Bb}	19.78 ^{Aa}	9.96 ^{ABc}	18.57 ^{Ba}	6.46 ^{Ae}	9.40 ^{Ac}	8.64 ^{Ad}	8.10 ^{Ad}
oLH + hTNF	13.15 ^{Bc}	19.21 ^{Ab}	10.16 ^{Ad}	21.87 ^{Aa}	5.94 ^{ABg}	9.16 ^{Ade}	7.52 ^{Bf}	8.61 ^{Ae}
oLH + mTNF	14.68 ^{Ab}	20.42 ^{Aa}	9.16 ^{Bc}	22.19 ^{Aa}	5.67 ^{Be}	9.95 ^{Ac}	8.01 ^{ABd}	8.87 ^{Ac}
Pooled SEM	0.15	0.15	0.16	0.16	0.15	0.19	0.17	0.15

*A,B,C,D Values in the same column and for the same steroid with different letters are significantly different (P≤0.05).

†^{a,b...g} Values in the same row with different letters are significantly different (P≤0.05).

‡Standard error of the mean.

reduced ($P \leq 0.05$) progesterone secretion 4, 18, and 24 hours after oviposition.

Co-treatment of F₃ granulosa cells with mTNF α and oLH increased ($P \leq 0.05$) progesterone production over that observed with oLH alone from HA, but not LA, hens as the ovulatory cycle progressed (Tables 12 and 13). This was evident at 4, 12, and 24 hours after oviposition in HA hens, regardless of age.

The decrease in F₃ pregnenolone secretion after treatment with hTNF α was primarily a result of lowered ($P \leq 0.05$) steroid production in younger hens at 4, 12, and 24 hours after oviposition in HA hens, and 4 and 12 hours after oviposition among LA hens (Tables 12 and 13). In older hens, this response to hTNF α was diminished and observed only in the LA line 18 hours after oviposition. Treatment of F₃ granulosa cells with mTNF α caused reduced ($P \leq 0.05$) pregnenolone secretion 24 hours after oviposition among both lines at 16 weeks of egg production, and at the same time in HA hens at 37 weeks of egg production.

The limited effect on pregnenolone secretion after co-treatment with mTNF α and oLH was found to occur early in the ovulatory cycle, and primarily in HA hens (Tables 12 and 13). Pregnenolone production in HA,

but not LA, hens increased ($P \leq 0.05$) at 12 hours after oviposition in younger hens, and at 4 and 24 hours after oviposition in older hens. Interestingly, mTNF α and oLH caused a reduction ($P \leq 0.05$) in pregnenolone production by F₃ granulosa cells from older LA hens 4 hours after oviposition. This was the only case in which the mTNF α and oLH treatment caused a decrease in either steroid (regardless of follicle size, age, or genetic line). Treatment of F₃ granulosa cells with hTNF α and oLH decreased ($P \leq 0.05$) pregnenolone secretion at 24 hours after oviposition in younger HA hens, and at 18 hours after oviposition in older LA hens.

4. DISCUSSION

As reported previously, Single Comb White Leghorn hens, selected for their ability to respond to an immune challenge with SRBC's, are known to differ in fertility, growth rate, age at puberty, egg production, and ability to resist bacterial or viral infection (Hall *et al.*, 1978; Gross *et al.*, 1980; Siegel and Gross, 1980; Siegel *et al.*, 1982; Martin *et al.*, 1990; Dunnington *et al.*, 1991). Siegel *et al.* (1982) and Bryan *et al.* (1997) reported that LA hens reached puberty before HA hens (13 and 14 days respectively), and laid more ($P \leq 0.05$) eggs to 300 days of age. In the current study, overall percent HDEP did not differ between genetic lines. However, both the date of puberty and 15% HDEP in hens of the LA line occurred 9 days prior to that of HA hens. Consequently, when percent HDEP to 442 days of age was measured, it was found that LA hens produced more ($P \leq 0.05$) eggs than HA hens. Dunnington and Siegel (1984) suggested that LA hens began to lay before HA hens simply because they attained the body weight necessary for reproduction at an earlier age.

Serial sampling of blood plasma revealed that levels of circulating progesterone and luteinizing hormone were higher ($P \leq 0.05$) among HA hens from the time of the preovulatory surge until ovulation. Given the

previously described differences between HA and LA hens, there may also exist a divergence in the levels and/or activities of certain enzymes within the steroid pathway. If these differences do exist, they could influence the production of steroids to the extent that the rate of egg production may be affected. Currently, the reason(s) for the differing levels of progesterone and luteinizing hormone between these genetic lines is unknown.

At both 16 and 37 weeks of egg production, the ovary from HA hens weighed more ($P \leq 0.05$) than that of LA hens. In older hens, this difference may be explained by the presence of larger ($P \leq 0.05$) preovulatory follicles (F_1 - F_3) and a greater ($P \leq 0.05$) number of small, 1-6 mm follicles on the ovary. In younger hens, however, an explanation was not as forthcoming. At 16 weeks of egg production, only F_2 follicles were larger ($P \leq 0.05$) in HA hens. There were no differences in the numbers of 1-12 mm follicles among HA and LA hens at 16 weeks of egg production. So, some factor other than tissue weight is contributory to the higher steroid production associated with HA hens.

The conversion of pregnenolone to androgens occurs via one of two different pathways. The Δ^5 pathway involves the metabolism of pregnenolone to androstenedione (A_4) using dihydroepiandrosterone

(DHEA) as an intermediary, while in the Δ^4 pathway, pregnenolone is converted to A_4 through progesterone (Lee *et al.*, 1998; Figure 3). A recent study showed that, although both avian granulosa and theca cells have functional Δ^4 and Δ^5 pathways, granulosa cells prefer the Δ^4 pathway, whereas theca cell steroidogenesis occurs mainly via the Δ^5 pathway (Lee *et al.*, 1998). As the F₁ follicle matures, its ability to convert progesterone to androstenedione diminishes, due to a decrease in the activity of the P450 17 α -hydroxylase/17,20 lyase enzyme complex (Robison and Etches, 1986). This inability of ovarian cells from the F₁ follicle to convert progesterone to

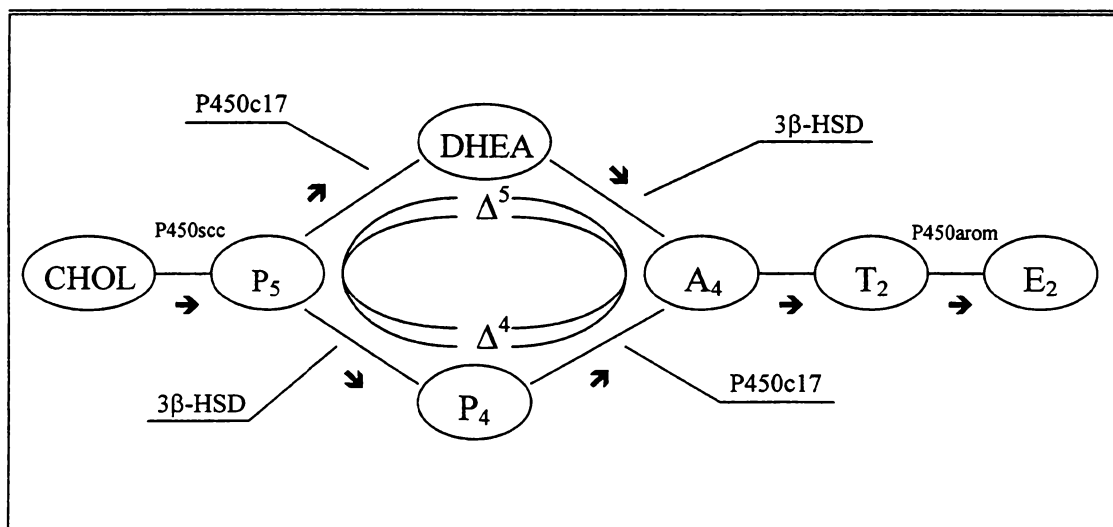


Figure 3. The Δ^4 and Δ^5 steroidogenic pathways*[†]

*CHOL: cholesterol; P₅: pregnenolone; P₄: progesterone; DHEA: dihydroepiandrosterone; A₄: androstenedione; T₂: testosterone; E₂: estradiol-17 β ; P450scc: cholesterol side-chain cleavage enzyme; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; P450c17: cytochrome P450 17 α -hydroxylase/17,20 lyase; P450arom: aromatase.

[†]This figure taken from Lee *et al.* (1998); Domestic Animal Endocrinology, 15:1-8.

androstenedione leads to an accumulation of the former. Progesterone released from granulosa cells in the hen activates receptors present in the hypothalamus and anterior pituitary, inducing the release of LH (Kawashima *et al.*, 1979). Luteinizing hormone, in turn, binds to receptors on the granulosa cells of preovulatory follicles, causing them to secrete more progesterone. This positive feedback loop culminates in preovulatory “spikes” (or peaks) of progesterone and LH that occur approximately 6 hours prior to ovulation (Wilson and Sharp, 1973; Johnson and van Tienhoven, 1980). Therefore, it is possible that a decrease in progesterone could interfere with this positive feedback cycle, and ultimately delay or inhibit ovulation.

The production of progesterone by granulosa cells increases as follicles mature. It is clear that much of the progesterone produced by granulosa cells from F₂ and F₃ follicles diffuses to the theca layer and is further metabolized to androgens or estrogen (Etches and Duke, 1984). Progesterone produced by F₁ follicle granulosa cells however is subject to no further modification, and is released into the peripheral circulation (Etches and Duke, 1984). In the current study, *in vitro* progesterone and pregnenolone production was higher among more mature preovulatory

follicles ($F_1 > F_2 > F_3$). However, at 37 weeks of egg production, F_2 follicles produced more progesterone and pregnenolone than did F_1 follicles. This unexpected difference in steroid production may have resulted from the inadvertent addition of higher cell numbers to culture media. Analysis of total media protein did not explain differences in steroid secretion because of the relatively high amount of BSA added to the culture media as compared to total protein secreted by granulosa cells in the 3 hour incubation period.

In the mammal, the effects of $TNF\alpha$ on ovarian steroidogenesis have been researched in detail. $TNF\alpha$ has been shown to inhibit FSH induced steroid production, aromatase activity, and LH receptor number in undifferentiated granulosa cells of the rat (Emoto and Baird, 1988; Adashi *et al.*, 1989; Darbon *et al.*, 1989). Given these observations, it is possible that the cumulus phenotype (i.e. low aromatase, reduced LH binding, and decreased steroidogenic potential) is caused by ovarian $TNF\alpha$ in mammals, and tends to “spare” the follicle from premature differentiation. Terranova (1997) suggested that one function of $TNF\alpha$ may be to protect the undifferentiated oocyte from the adverse effects associated with estrogen. Butcher *et al.* (1975) reported that delayed ovulation, which is associated

with prolonged exposure to estrogen, increased abnormalities in oocytes. More over, FSH driven estrogen production and LH receptor formation are of critical importance if granulosa cells are to differentiate (Darbon *et al.*, 1989). Therefore, TNF α may be involved in the inhibition of this process in mammals.

In mammalian granulosa cells that are undergoing or have undergone differentiation to luteal cells, the effects of TNF α appear to be somewhat different. TNF α has been shown to reduce LH stimulated progesterone production in the rat, pig, and cow, while stimulating the production of PGF_{2 α} (Velhuis *et al.*, 1991; Benyo and Pate, 1992; Adashi *et al.*, 1990; Bränströmm *et al.*, 1993; Pitzel *et al.*, 1993). Therefore, it appears that TNF α may participate to some extent in luteal regression (Terranova *et al.*, 1995).

There is very little information available concerning the effect of cytokines on ovarian steroidogenesis in the avian. Bryan *et al.* (1997) reported that *in vitro* progesterone was consistently reduced ($P \leq 0.05$) below basal levels when granulosa cells from domestic hens were treated with a dose of 5 ng/ml hTNF α .

In this study, treatment of granulosa cells with human TNF α decreased ($p \leq 0.05$) basal progesterone production in F₁ and F₃ follicles, and confirmed the report of Bryan *et al.* (1997). Similarly, basal pregnenolone secretion from all preovulatory follicles was reduced ($P \leq 0.05$) in response to treatment with hTNF α . However, in most cases, preovulatory follicles appeared unresponsive to mouse TNF α , with few effects observed on basal progesterone and pregnenolone secretion. Johnson and van Tienhoven (1984) demonstrated a dose-dependent decrease of *in vivo* progesterone levels in response to injection with aminoglutethimide (a potent inhibitor of steroidogenesis). This reduction in progesterone resulted in the absence of either an LH surge or ovulation. The reintroduction (via injection) of progesterone resulted in a renewed preovulatory surge of LH, followed by ovulation of the F₁ follicle. While this is proof that a reduction in progesterone can inhibit ovulation, the threshold at which progesterone must fall below for this to occur is unknown.

In order to determine the point during the ovulatory cycle when TNF α was affecting steroid secretion, a time-course study was conducted. Although hTNF α consistently caused a numerical reduction in basal

progesterone and pregnenolone secretion by granulosa cells, these differences were rarely significant ($P \leq 0.05$). The lack of significance observed in the time-course study may have been due to small sample size (5 hens/time-point). As a result, no clear pattern emerged as to when specifically hTNF α was having its affect.

It is well known that LH stimulates the production of progesterone from granulosa cells in the domestic hen (Hammond *et al.*, 1980; Marrone and Hertelendy, 1983). Asem *et al.* (1983) determined that progesterone production by granulosa cells increased when cultured in the presence of ovine LH. In the current research, treatment of granulosa cells with oLH stimulated ($P \leq 0.05$) progesterone production over basal levels without exception.

In contrast to the effects of hTNF α on basal progesterone production during the ovulatory cycle, there was indeed a clear and well defined response of granulosa cells when treated with mTNF α , but not hTNF α , in the presence of an ovulatory amount of oLH (100 ng/ml). Under these circumstances, the secretion of progesterone and pregnenolone increased (over that produced in response to treatment with oLH alone) among hens of both ages and genetic lines, in all follicle sizes, and at almost all times

during the ovulatory cycle. Co-treatment of granulosa cells with hTNF α and oLH did, occasionally, increase ($P \leq 0.05$) progesterone and pregnenolone secretion over that of oLH alone. However, this was not as consistent as that observed after treatment with oLH and the murine form of TNF α . Bränström *et al.* (1995) reported that co-treatment of perfused rat ovaries with hTNF α (40 ng/ml) and oLH (100 ng/ml) increased *in vitro* progesterone production as compared to treatment with oLH alone. Furthermore, they reported a four-fold increase in the rate of LH induced ovulatory rates when perfused ovaries were exposed to hTNF α . At this time, the significance of an increase in progesterone productions by granulosa cells in the hen is not known. Zolti *et al.* (1990) found that concentrations of TNF α in bovine follicular fluid increased as ovulation approached. This implies the possibility that, in the hen, TNF α may potentiate the LH induced production of progesterone as follicles mature, and thereby augment or at least influence the ovulatory process.

Recently, Tartaglia and Goeddel (1992) proposed a model by which the 75 kDa type II receptor for TNF α could sequester TNF α and “pass it on” to the 55 kDa type I receptor. As discussed previously, the type II receptor has a greater affinity for TNF α than does the type I receptor.

However, TNF α dissociates from the type II receptor at a greater rate than from the type I receptor, thereby facilitating interaction of the cytokine with the type I receptor (Medvedev et al., 1996). Since the type II receptor does not associate with human TNF α , this effect would only be observed when studying the murine form. Corroborating evidence for this “ligand passing” model comes from a study in which hTNF α was considerably less effective than mTNF α in the induction of IL-6 *in vitro* and *in vivo* in the mouse (Benigni *et al.*, 1996). However, when the authors injected antibodies to each receptor, they found that activation of the type I receptor was more effective in stimulating the production of IL-6. In fact, it appeared that the antibody to the type II receptor was absolutely incapable of inducing IL-6 production (Benigni *et al.*, 1996). Recently, the type I receptor for TNF α has been implicated in the induction of HIV-1 expression (Matsuyama et al., 1991). Butera et al. (1996) found no direct action of HIV-1 by the type II receptor. However, when the type II receptor was blocked (by antibodies), the effect of TNF α on HIV-1 induction was decreased markedly, suggesting to the authors that the type II receptor enhanced the binding of TNF α to the type I receptor. There exists the possibility that this “ligand passing” model is applicable to the

increased *in vitro* production of progesterone by granulosa cells of the hen in response to mTNF α and oLH. It should not, however, be ruled out that the effects of mTNF α and oLH on steroidogenesis in the laying hen are the result of a direct activation of the type II TNF α receptor.

Future research should be aimed at the identification and localization, as well as the physiologic roles, of the TNF α receptors in the ovary of the laying hen. Finally, since these studies utilized only granulosa cells, it would be of great interest to study the effect(s) of TNF α on the intact follicle composed of theca and granulosa cells.

5. SUMMARY

Single Comb White Leghorn hens, genetically selected for either high or low levels of antibody in response to an immune challenge with SRBC's, displayed different production characteristics. Low antibody hens reached sexual maturity approximately 9 days before HA hens, and laid more ($P \leq 0.05$) eggs to 442 days of age. Analysis of *in vivo* steroid production showed that both progesterone and luteinizing hormone were higher ($P \leq 0.05$) among HA hens during the 6 hours prior to ovulation. Although the reason(s) for these differences are currently unknown, they may be due to differences in the levels or activities of enzymes within the steroid pathway or differential tissue response to either gonadotropins or steroids.

In vitro progesterone and pregnenolone production was generally higher ($P \leq 0.05$) among HA hens as compared to LA hens, and in larger versus smaller preovulatory follicles. As in the mammal, $\text{TNF}\alpha$ appears to inhibit basal steroidogenesis in the laying hen. Treatment of granulosa cells with h $\text{TNF}\alpha$, but not m $\text{TNF}\alpha$ resulted in reduced ($P \leq 0.05$) progesterone and pregnenolone production. However, these decreases were not as prevalent, or of the magnitude of those previously reported by our laboratory (Bryan *et*

al., 1997). Conversely, *in vitro* treatment of granulosa cells with mTNF α in the presence of an ovulatory amount of oLH (100 ng/ml) consistently and markedly increased ($P \leq 0.05$) the secretion of progesterone and pregnenolone over that observed after treatment with either oLH alone or hTNF α and oLH.

The observation that co-treatment of granulosa cells with mTNF α and oLH stimulated steroid secretion over that produced in response to treatment with hTNF α and oLH suggests a role for the type II TNF α receptor in potentiating the effects of the type I TNF α receptor. However, since the intracellular domains of the two receptors share little or no sequence homology, it is unclear whether these differences in steroid production are the result of ligand passing, or a direct activation of the type II receptor.

Due to the high incidence of exposure to microbial pathogens in commercial poultry operations, the study of the effects of cytokines on avian reproduction is of great importance. A better understanding of how these cytokines influence the reproductive cycle in the avian will allow for appropriate responses to infection, as well as, reinforce the need for preventative measures.

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