EFFECTS OF CYTOKINES ON ESTRADIOL PRODUCTION BY BOVINE GRANULOSA CELLS IN VITRO: DEPENDENCE ON SIZE OF FOLLICLE

By

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1993

OKLAHOMA STATE UNIVERSITY

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Leon Spicer for his advise, friendship and continuous support during my studies at Oklahoma State University.

Also, I want to express my gratitude to Dr. Rodney Geisert and Dr. Tim Braden, members of my thesis committee for their valuable suggestions while reviewing this work.

My gratitude to Rob Vernon, Jorge Vizcarra, and collaborators for their friendship, computer advice, and laboratory assistance during the research.

I would like to express my sincere gratitude to the best roommate and friend I have ever had Gloria Gadea-Lopez, for her moral support and encouragement to continue with the graduate program. Also, to my dear friend Brenda de Rodas, who has had the patience to listen and to understand me in my most problematic situations during my stay in Stillwater.

To my other dear friends Dr. Georges Sabbagh and Norma Ruiz for their friendship and collaboration to the cause. To these and many other people that have helped me during all these years, my sincere gratitude.

I am deeply grateful to my parents Elias Alpizar and Maria Luisa Montero for their understanding, patience, and sacrifice. To my brothers and sisters, for their love, great support and encouragement to finish this task.

I want to dedicate this thesis to my dear big family and specially to all the women who have struggled to learn and to contribute to society.

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CHAPTER I

INTRODUCTION

The hemopoietic system is regulated by many different humoral factors that are produced by blood cells such as macrophages, T and B lymphocytes, granulocytes, eosinophils, and mast cells, which are all capable of self renewal and differentiation. These humoral factors are glycoproteins that serve as cell mediators in immune response, inflammatory reactions, and hemopoiesis (Miyajima et al., 1992). These glycoproteins are collectively called cytokines which are not only involved in the immune response system but also affect the reproductive system. The biological activity of cytokines may differ a great deal among each other and also may overlap with each other. Depending on their chemical structure, cytokine receptors can be shared by more than one cytokine or they can be very specific. Receptors have been found in many types of cells and these receptors can have high or low affinity for a specific cytokine. Receptors and mRNA for different cytokines can be found in the ovary. This suggests a role in ovulation since ovulation can be considered as an inflammatory reaction. In addition, it has been hypothesized that the presence of cytokines in the ovary can affect steroidogenesis.

Based on this hypothesis many studies have been conducted

and subsequently the presence of blood cells (i.e., macrophages) in follicular fluid and in several follicular cells from different species has been reported. For instance, regulatory effects of interleukin-1 in rat (Adashi et al., 1989), human (Barak et al., 1992a), and porcine (Fukuoka et al., 1989) granulosa cells have been reported. Other cytokines are produced by thecal and interstitial cells and exert an autocrine and paracrine effect. There have been no reports on the effect of cytokines relative to size of follicle in any species, and few reports on the effect of cytokines on bovine granulosa cell function. Therefore, the objective of these experiments was to determine the effects of some cytokines on in vitro estradiol production by granulosa cells obtained from small and large bovine antral follicles.

CHAPTER II

REVIEW OF LITERATURE

Role of Cytokines in the Immune and Inflammatory Reaction

The immune system is composed of a network of interactions between cells that must communicate with each other for an effective immune response to occur against a stressful agent such as viral infections or more complex diseases. The non-self immune response has been known as the nonspecific antimicrobial system for self-limiting inflammation. The major histocompatibility gene complex (MHC) codes for cell surface glycoproteins necessary for the immune response. There are two classes of MHC. Class I MHC glycoproteins are found on all nucleated cells. Class II MHC glycoproteins are found in macrophages and B lymphocytes, as well as in granulosa cells in the ovary (Hill et al., 1990) in response to treatment with macrophage product (IFN-γ) in vitro (Benyo et al., 1991). Recent studies have shown that the defense mechanism can be influenced by substances produced by cells involved in the immune response (Balkwin and Burk, 1989). Such substances, now called cytokines, are proteins or glycoproteins that can affect the cells that produce them or can affect other

nearby cells. In other words, they can have an autocrine or paracrine effect in the cells involved in the immune response. Their primary structures consist of several hundred amino acids with N-glycosylations except for tumor necrosis factor α (TNFα; Harrison and Campbell, 1988). The cytokine family consists of interleukins (IL 1 to 12 and with α and ß structures), interferons (IFN-α,ß and γ), tumor necrosis factors (TNF α and ß), granulocyte-macrophage colony stimulating factor (GM-CSF), and others have been identified. Cytokines are produced by monocytes, activated macrophages, T cells, B cells and fibroblasts (Babiuk et al., 1990), and their target cells are of wide variety. Harrison and Campbell (1988) categorized cytokine action in six ways: 1) T cell activation, growth and differentiation (IL-1, IL-2, IL-4, IL-5, IL-6, IFN-γ), 2) B cell activation, growth and differentiation (IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IFN-γ), 3) hematopoiesis (CSFs, IL-1, IL-3, IL-4, IL-5), 4) inflammation (CSFs, TNFs, IFN-γ, IL-1, IL-3, CSFs), 5) mitogenic and metabolic effects on non-immune cells (TNFs, IFN-γ, IL-1, IL-6) and 6) cytotoxicity (TNFs, IFN-γ, IL-1). A cytokine can enhance or inhibit the synthesis of other cytokines. For instance, IL-1 and TNF stimulate each others release and these two stimulate IL-6 release from monocytes and macrophages (Nathan, 1987; Brouckaert et al., 1989). IFN-γ has been recognized as one of the exogenous stimulatory signals of TNF receptor expression (Aggarwal et al., 1985). On the other hand, IL-10 which is a T cell line-

derived cytokine, inhibits IL-1 and TNF production (Ralph et al., 1992). Also, it has been reported that IL-10 inhibits the expression of IL-6, IL-8 , GM-CSF, and class II MHC in monocytes stimulated by IFN's (de Waal Malefyt et al., 1991).

Even though there are differences in the structure of cytokines, their actions appears to overlap in some cases. For instance, TNF and IL-1 (Nathan, 1987; Spinas et al., 1990) work synergistically in the activation of T and B cells, as well as monocytes, macrophages and other cells. IL-12 can enhance the induction of IFN-γ production by resting or activated peripheral blood natural killer (NK) cells and T cells (Chan et al., 1991). IL-12 and IFN-γ suppress T cell dependent synthesis of immunoglobulin E stimulated by IL-4 in vitro (Kiniwa et al., 1992). Also, IL-12 works synergistically with IL-2 in the generation of cytotoxic T cells and lymphokine-activated killer cells (Gubler et al., 1991).

The concentration of cytokines in blood of normal animals is minimal. However, it is expected that their concentrations will increase during an immune reaction. For example, Ertel et al., (1991) measured the concentrations of IL-1 in plasma from mice under normal conditions, sham surgery, and cecal ligation and puncture. He found that untreated animals had an IL-1 concentration of 0.04 U/ml, while those animals that were treated had 0.42 U/ml and 15.53 U/ml for sham surgery and ligation-puncture,

respectively, showing an increase in plasma IL-1 occurs after an immune reaction. The same increasing tendency was observed for IL-6, having its maximum level of 0.83 U/ml, 5.4 U/ml, and 62.1 U/ml for unmanipulated animals, sham surgery, and ligated-punctured animals, respectively. Another study reported the same increasing pattern of IL-6 when measured in horse plasma, having much higher levels (10,128 U/ml) after 1000 ng of endotoxin/kg was applied I.V. to the horses (MacKay and Lester, 1992). Other studies, where IL-6 activity was tested in normal human beings or rodents, failed to detect circulating IL-6 activity (Ida et al., 1990; Van Gool et al., 1990; Ayala et al., 1991).

TNF levels in peripheral circulation were undetectable when measured in normal mice (Ertel et al., 1991), horses (MacKay and Lester, 1992) and humans (Beutler, 1988). When the animals or individuals underwent surgery or were treated with endotoxin, the levels increased dramatically after 2 hrs of treatment (Lahdevirta et al., 1988, Beutler, 1988; Ertel et al., 1991; MacKay and Lester, 1992). In general, it appears that the body will react to a foreign agent through the immune response by producing cytokines in increasing levels.

Biology of Cytokines

This review will only cover the biology of IL-1, IL-2, IL-6, IFNs, and TNFs and their receptors.

Interleukin-1

IL-1 is a member of a group of immune mediators, secreted by a variety of immune and nonimmunocompetent cells (Lomedico et al., 1984; Dinarello, 1989). Two forms of IL-1 have been cloned; IL-1ß was cloned from human blood monocytes stimulated by endotoxin (Auron et al., 1984) and IL-1α was cloned from the mouse macrophage line P388D (Lomedico et al., 1984). Subsequent to these findings, IL-1ß has been cloned in the cow, rabbit, rat and mouse and IL-1α has been cloned in human, rat, and rabbit (Dinarello, 1989). There is 26% amino acid homology within the two human IL-1 forms, and 25% of amino acid homology among the various animal species (Dinarello, 1989).

In vitro, not only hematopoietic cells produce IL-1, but also endothelial cells, keratinocytes, smooth muscle cells, and renal mesangial cells transcribe large amounts of IL-1 mRNA and translate IL-1 protein (Barak et al., 1986; Auron et al., 1987; Dinarello, 1989; Deyerle et al., 1992). The control of IL-1 translation is affected by other cytokines. IL-1-induced IL-1 production (either IL-1ß-induced IL-1α, or IL-1α-induced IL-1ß) is suppressed by IFNs, whereas IFN-γ enhances IL-1 synthesis after endotoxin or TNF stimulation (Ghezzi and Dinarello, 1988). It has been proposed that IL-1 synthesis in monocytes is autoregulated not only by cytokines such as TNF, IL-1, and IFN-γ but also by prostaglandins produced by the same cells through a negative feedback mechanism (Favrot et al., 1989;

Sung and Walter, 1991). Similarly, Knudsen et al., (1986) observed that IL-1 synthesis is suppressed when murine cells were treated with prostaglandin E_2 (PGE_2). On the other hand, IL-1 induced the release of PGE_2 mediated via induction of prostaglandin synthetase synthesis in a concentration and time dependent manner (Raz et al., 1988; Bull and Dowd, 1992).

In addition to the inductive effect of IL-1 on PGE_2, IL-1 acts as a lymphocyte and thymocyte activating factor, proteolysis activating factor, mononuclear cell-activating factor, and may count for a majority of observed acute-phase changes in patients with bacterial infection, injury or chronic inflammatory disease (Favrot et al., 1989; Dinarello, 1989). IL-1 also enhances T cell proliferation, augments the response of T, B and NK cells, stimulates the production of IL-6, IFN-γ, and IL-2 by T cells, and augments the expression of IL-2 receptors in murine thymocyte cells (Dinarello, 1989).

Holtmann and Wallach, (1987), found that IL-1 decreased the vulnerability of the cells to the cytolytic effect of TNF by inducing a decrease in TNF receptors in human foreskin fibroblasts and SV80 and HeLa cell lines. On the contrary, Fischer et al., (1992) stated that IL-1 contributes to and potentiates TNF-α induced cardiovascular collapse in baboons through their synergistic effect. Reasons for this discrepancy are unclear but may be due to different effects of IL-1 depending on the type of cell,

culture system, species, or the methods used to analyze the cytokines.

It is thought that IL-1 may play an important role in reproduction since its presence has been documented in the human (Khan et al., 1980) and porcine (Takakura et al., 1989). Furthermore, it has been revealed that IL-1 possess potent antigonadotropic (Fukuoka et al., 1989; Gottschall et al., 1989; Yasuda et al., 1990), or steroidogenic (Nakamura et al., 1990) properties. In addition to these properties, IL-1 has been established as a mediator of inflammation (Dinarello, 1989) and since ovulation may constitute an inflammatory-like reaction, IL-1 may play an intermediary role in the ovulation process (Nothnick and Pate, 1990; Hurwitz et al., 1991; Bull and Dowd, 1992; Kokia et al., 1992). But the importance of the effects of IL-1 likely depends on the level of IL-1 receptors found in a particular cell type.

Studies on IL-1 receptors have focused on two cell types: murine thymoma cells and fibroblasts. Two types of receptors (I and II) have been found. Type I IL-1 receptors are present on T cells, B cells, fibroblasts, epithelial cells, and keratinocytes. Evidence indicates that type I IL-1 receptors are subject to up-regulation by forskolin and are cAMP dependent (Bonin et al., 1990; Hurwitz et al., 1992) but this dependency is not yet clear. Type II IL-1 receptors are present on B cells and macrophages (Hurwitz et al., 1992). The molecular structure of type II IL-1

receptor belongs to the immunoglobulin superfamily (Dinarello, 1989). The IL-1 receptors are specific in that they do not recognize other cytokines. However, these receptors do not distinguish between IL-1α or IL-1ß (Dinarello, 1989; Miyajima et al., 1992). Recent studies have found that the expression of both types of IL-1 receptors are abundant in nonlymphoid organs such as brain, pancreas, uterus, and skin (McMahan et al., 1991; Deyerle et al., 1992). In addition, IL-1 receptor transcripts were detected in RNA from whole ovaries and follicular aspirates taken from women undergoing in vitro fertilization and embryo-transfer. Later, Hurwitz et al., (1992) found that mRNA for IL-1 receptors was detectable in cultured human granulosa and thecal cells. Because of the multiple effects of IL-1 on ovarian cells it is thought that IL-1 receptors are present in murine (Gottschall et al., 1987; Kasson and Gorospe, 1989) and porcine (Fukuoka et al., 1989; Yasuda et al., 1990) granulosa cells.

Interleukin-2

IL-2 is secreted by T lymphocytes and was first identified as a T cell growth factor (Morgan et al., 1976; Robb, 1984), which allows the clonal expansion in vitro of T cells activated by antigens. The induction of IL-2 in T cells and T cell lines consist of a synergistic effect of IL-1 with antigens, mitogens, ionophores, or activators of protein kinase C (PKC) through two induction

signals. The first signal involves an increase in cytosolic calcium, and the second signal involves increase in the PKC activity (Dinarello,1988; Oyaizu et al., 1992).

IL-2 is one of the most powerful activators of human monocytes in vitro which respond to IL-2 by producing hydrogen peroxide (Holter et al., 1987). Also, IL-2 induces TNF-α mRNA, expression of IL-1ß mRNA, microbial and tumoricidal activity, and IL-6 mRNA and IL-6 induction in human monocytes (Musso et al., 1992). IL-2 has a key role in the induction of immune response by activating T lymphocytes, NK cells, B lymphocytes, and in the production of other cytokines (Harrison and Campbell, 1988). IL-2 is also capable of the stimulating lymphokine-activated killer cells (LAK) which are able to destroy specific tumoral cells without damaging normal cells through a mechanism called "non-MHC restricted" (Grimm et al., 1982; Hatakeyama et al., 1989). Because of its function in the immune system, IL-2 has been widely used in the treatment of cancer patients. There are endogenous factors that induce IL-2 synthesis (IL-1, IL-5, and IL-6), as well as factors that inhibit the induction of receptors for IL-2 on T cells therefore limiting the effect of IL-2 (Naito, 1988; Hatakeyama et al., 1989).

IL-2 receptors have been characterized on resting T cells, NK cells, and some B and T cell lines (Espinoza-Delgado, 1992; Hatakeyama et al., 1989). There are two subunits of the IL-2 receptor: p55 (α) and p75 (ß) kDa

glycoprotein, having low and medium affinity, respectively in accordance to binding ability to IL-2. These two combine to form a high affinity receptor (αß). IL-2 regulates IL-2 receptor ß mRNA, and IFN-γ induces IL-2 receptor α mRNA (Naito et al., 1989; Hatakeyama et al., 1989). Also, IL-1 is considered one of the factors promoting the expression of IL-2 receptors (Nathan, 1987). It has been shown that the intermediate and high affinity IL-2 receptors can both internalize IL-2 into the cell (Hatakeyama et al., 1989).

Interleukin-6

IL-6 is a protein of 184 amino acids with potential N-glycosylation sites and 4 cysteine residues, showing a significant homology (25%) with granulocyte-colony stimulating factor, a growth factor involved in the immune response (Hirano et al., 1986), suggesting they may have some functional similarities. Comparison of the cDNA sequences of human IL-6 and murine IL-6 shows a homology of 65% at the DNA level and 42% at the protein level (Tanabe et al., 1988). IL-6 is secreted by T lymphocytes, B lymphocytes, monocytes, fibroblasts, keratinocytes, endothelial cells, granulosa cells and several tumor cells (Kishimoto, 1989; Harrison and Campbell., 1989; Gorospe et al., 1992). IL-6 production in T cells is induced by T cell mitogens and by antigenic stimulation in the presence of direct contact with macrophages (Helfgott et al., 1987). Its production is also enhanced by lypopolysaccharides (LPS)

(Horii et al.,1988), IL-1, tumor necrosis factor (TNF), and INF-ß (Kishimoto, 1989). Glucocorticoids negatively regulate IL-6 gene expression in various cells types (Helfgott et al., 1987), and immunomodulatory eicosanoid PGE_2 is involved in the regulation of IL-6 release during sepsis which is the presence of pathogenic organisms in the blood (Ertel et al., 1991).

IL-6 plays an important role in the final differentiation of B cells into antibody producing cells (Hirano et al., 1985; Muraguchi et al., 1988). There is also evidence that IL-6 induces the expression of IL-2 receptors (Noma et al., 1987), induces IL-2 production in mitogen-simulated T cells and thymocytes (German et al., 1987) and up-regulates IL-3 receptor expression in murine spleen cells (Kishimoto, 1989). In the presence of human and murine thymocytes, IL-6 induces proliferation and cytotoxicity of T cells (Takai et al., 1988; Okada et al., 1988). In the systemic reaction of inflammation or injury called acute phase response, IL-6 is involved in the induction of the production of several proteins in vivo such as fibrinogen, alpha-1-antichymotrypsin, and alpha-1-acid glycoprotein (Nijtein et al., 1987) showing a synergistic effect with glucocorticoid (Castell et al., 1988) on the induction of acute phase proteins. In vivo studies with rats demonstrated that the application of IL-6 caused an increase in the plasma levels of adrenocorticotropic hormone (Naito et al., 1988) through stimulating the release of

corticotropin-releasing hormone. Also, there is evidence that IL-6 is involved with the nerve growth factor system (Kishimoto, 1989). Therefore, IL-6 appears to be involved with the immune response as well as the neuro-endocrine system of the body. In general, IL-6 provides multiple signals on various tissues and cells that can be divided into three categories: 1) induction of differentiation or specific gene expression such as Ig induction in B cells or acute phase proteins; 2) stimulation of cell growth; and 3) inhibition of cell growth. Why IL-6 provides such a variety of signals is unclear but this may be related to the differential expression of receptor levels on various cell types.

The IL-6 receptor consists of two polypeptide chains, a ligand binding chain and non-ligand binding, signal transduction chain having a sequence homology to the Ig superfamily and having no tyrosine kinase domain. The detectable number of IL-6 receptors ranges from 100 to 20,000 sites/cell depending on the cell type (Taga et al., 1987; Kishimoto, 1989). IL-6 receptors are expressed in various cells such as activated B cells, resting T cells, B lymphoblastoid cell lines, myeloma cell lines, hepatoma cell lines, and monocytes cell lines (Kishimoto, 1989; Miyajima et al., 1992). The overall amino acid homology between human and mouse IL-6 receptor is 54% (Sugita et al., 1990).

Interferons (IFNs)

Interferons are proteins with a molecular weight of 16-27 kDa (Pestka et al., 1987). These proteins are classified as type α, type ß, and type γ according to their antigenicity. IFN-α and IFN-ß were identified as products of virus-infected cells such as leukocytes and fibroblasts, whereas IFN-γ is produced as primary response of T lymphocytes to mitogenic stimulation (Morris, 1988; Xiao and Findlay, 1991). IFN-γ is considered to be structurally different from the IFN-α and IFN-ß (Gray and Goeddel, 1982). Human IFN-α is part of a family of multifunctional cytokines consisting of at least 15 subtypes with 78-98% amino acid homology among the subtypes (Overall and Hertzog, 1992) and there is 45-75% homology of IFN-α between species (Roberts, 1989). Human IFN-α has considerable variation in its specific activity on human cells, and has only a weak cross-reaction on mouse cells (Rehberg et al., 1982). In cattle, a pregnancy specific protein (bTP-1), produced by the conceptus during the first days of pregnancy, is a member of the 172 amino acid IFN-αII family (Imakawa et al., 1989; Stewart et al., 1990)

IFNs have multiple activities including antiviral, antiproliferative, and differentiative activity (Petska et al., 1987). IFNs have been shown to modulate the activity of T and B lymphocytes, NK cells, macrophages, and other cells involved in the immune response (Hill et al., 1979; Capon et al., 1985; Billiau, 1988). IFNs are known to

inhibit the growth of tumoral cells (Wallach et al., 1982; Pace et al.,1983; Lee et al., 1986) as well as normal cells. IFNs induce the production of ILs and increase TNF activity (Tsujimoto et al., 1986), which may be related to other effects and not directly to IFN. IFN-α was first found in T cells (Gutterman, 1982) and in chronic myeloid leukemia by Talpaz et al. (1986). IFN-α shares some sequence homology at both the amino acid and nucleotide level with approximately a 25% amino acid identity to IFN-ß.

It has been shown that a systematic administration of IFN-α and IFN-γ can inhibit lipopolysaccharide-induced reaction, which is an immune reaction that causes the manifestation of increase in temperature, inflammation or shock, suggesting that IFNs down regulate inflammatory responses that could be detrimental to the animal (Billiau, 1987). IFN-γ also activates eosinophils and macrophages (Lambert and Paulnock, 1989). IFN-γ produced by murine T helper-1 cells suppressed the IgE-mediated allergic response that other cytokines enhanced (Mosmann and Coffman, 1989). Also, IFN-γ treatment increased the number of available TNF receptors without affecting the apparent affinity and stimulated synthesis of mRNA for the TNF receptor protein (Tsujimoto and Vilcek, 1986; Tsujimoto et al., 1986) in human HeLa cell cultures. IFN-γ increased the sensitivity of human cancer cells to TNF (Ruggiero et al., 1986; Brouckaert et al., 1989), and consequently suggests a synergistic cytotoxic effect with TNF. Local application of

IFN-γ intensifies the response within an inflammatory focus and appears to have an important role in the regulation of acute phase response and uncontrolled inflammatory reactions, which may lead to pathological alterations to host tissue (Sordillo and Peel, 1992). The presence of IFNs in several organs of normal individuals suggests that IFNs are important in normal physiology (Tovey et al., 1987), especially in maintaining pregnancy (Roberts, 1989). There is also evidence that IFNs may play an important role in modulating the ovarian function because of their effects on ovarian cells (Orava et al., 1985; Gorospe et al., 1988; Xiao and Findlay, 1991).

Competitive binding of human IFNs on human cells seems to indicate that all human IFN-α and IFN-ß share a common receptor, while IFN-γ is recognized by a distinct receptor system (Merlin et al., 1985; Uze et al., 1990; Miyajima et al., 1992; Senda et al., 1992). There is no homology between IFN-γ and IFN-α or IFN-ß. The number of IFN receptors is usually in the order of 10^2 to 10^3 per cell, which is about 100-fold less than that for hormones or growth factor receptors (Kishimoto, 1989). IFN receptors are present in all tissues and even on the surface of most interferon-resistant cells (Aguet and Mogensen, 1983). IFN receptors are species specific, and therefore, each species may have its own specific signal transduction (Miyajima et al., 1992).

Tumor Necrosis Factor (TNF)

TNF is a polypeptide with a molecular weight of 17 kDa (Beutler and Cerami, 1987). Two forms of TNF have been identified: TNF-α and TNF-ß which have a 28% amino acid homology (Sherry and Cerami, 1988). TNF-α is produced by activated macrophages, and TNF-ß (or lymphotoxin) is produced by T lymphocytes (after stimulation by specific antigens), endothelial cells, and keratinocytes (Aggarwal et al., 1985; Beutler and Cerami, 1987; Favrot et al., 1989). TNF-α is thought to play a major role in the cachexia which is a general emaciation occuring in the course of a chronic infection and the physiopathologic manifestation of shock produced by sepsis (Schmid et al., 1985; Beutler et al., 1985; Dinarello, 1989; Favrot et al., 1989), as well as anorexia, diarrhea, hypertension and disseminated intravascular coagulation that may cause death (Bielefeldt et al., 1984; Waage et al., 1987; Lantz et al., 1991). TNFs induce the synthesis of monokines such as IL-1ß and IL-6 which also have been implicated in the pathophysiology of infections (Dinarello et al ., 1988). The effect of TNF or IL-1 on a variety of cells in vitro as well as systemic effect in vivo are often biologically indistinguishable because of their synergistic action (Dinarello, 1989). TNF also synergizes with IFN-γ in their cytotoxic action on tumor cells (Tsujimoto et al., 1986). TNF activates

eosinophils and therefore plays a role in the response of parasitic infection (Favrot et al., 1989). Also, it has been documented that TNF stimulates the Na^+/H^+ antiporter in human fibroblasts causing intracellular alkalinization and further cell proliferation (Yiul-Lee et al., 1992). Production of TNF has been shown to be down-regulated by PGE_2 through a negative feedback mechanism during sepsis (Ertel et al., 1991). Like the other cytokines, TNF is also involved in the endocrine system, modulating steroidogenesis in the ovary of various species (Adashi et al., 1990; Fukuoka et al., 1992). All these functions of TNF are thought to be mediated by specific binding sites or receptors.

Two different receptors for TNF have been found: a 55 kDa and a 75 kDa protein, expressed at low levels in various murine cells. Both receptors bind TNF-α and TNF-ß equally well (Miyajima et al., 1992). The amino acid homology between the human and murine TNF receptor is 75% (Harrison and Campbell, 1989). About 1000 receptors per cell were reported in binding assays with L cells and murine TNF (Kull et al., 1985). High affinity TNF receptors are present in a variety of normal and transformed tumor cells (Miyajima et al., 1992). TNF receptors are also present in porcine granulosa cells (Veldhuis et al., 1991).

TNF receptors appear to be up-regulated by other cytokines such as IFN-γ in human HeLa D98 cell cultures (Ruggiero et al., 1986; Tsujimoto et al., 1986). Studies

done with rat cell cultures reported that the three forms of IFN (α, β and γ) increased TNF receptor numbers without affecting their affinity (Scheurich et al., 1986; Ruggiero et al., 1986; Tsujimoto and Vilcek, 1986; Scheurich et al., 1989). It was also reported that IL-1ß as well as tumor-promoting phobol diester can down-regulate TNF binding by decreasing the number of TNF receptors (Holtmann and Wallach, 1987; Holtmann et al., 1991; Bethea et al., 1992). This regulation might be modulated by the number of IL-1 receptors or by enhancing phosphorylation of protein kinase C or other types of proteins. Scheurich et al. (1989) identified protein kinase A (PKA) as one of the endogenous regulatory elements that control TNF receptor expression by a mechanism independent of and antagonistic to the previously described PKC-mediated control of receptor affinity, enhancing TNF receptor synthesis.

Presence of Immune Cells in the Ovary

As reviewed above, it has been shown that white blood cells such as macrophages, T lymphocytes and others, under stimulation or activation, produce certain substances called cytokines which are related to the immune response and inflammatory reactions. It was suggested that a modified inflammatory process was involved in ovulatory rupture of the follicular wall (Lipner, 1988; Espey, 1992). White blood cells have been viewed as moderators of acute inflammatory response and the presence of white blood cells

in the ovary has been reported (Nakamura et al., 1987; Katabuchi et al., 1989; Loukides et al., 1990; Gaytan et al., 1991; Fukumatzu et al., 1992; Brannstrom et al., 1993). Historically, macrophages have been considered for their phagocytic activity during ovulation, follicular atresia, and luteal regression (Lobel and Levy, 1968; Leavitt et al., 1973; Bagavandoss et al., 1988). In addition to their phagocytic role, macrophages secrete a wide variety of products, including growth factors, arachidonic acid metabolites, neutral proteases, and some cytokines. Not only macrophages produce cytokines, but also white blood cells that infiltrate the follicle or corpus luteum (Standaert et al., 1991). Any of these secretory products could influence ovarian function by regulating granulosa, thecal, and luteal cell growth, and/or steroidogenesis.

Ovarian Follicles

The presence of macrophages in the ovary has been documented by several authors. These macrophages likely produce cytokines that may act as autocrine or paracrine modulators of ovarian cell functions. Loukides et al., (1990) found that 5 to 15% of the resident nucleated cells in human follicular fluid were monocytes and macrophages and that there appears to be a stable population of these cells within the microenvironment of the ovarian follicle during the follicular and luteal phases. Katabuchi et al., (1989) have suggested that in the small antral follicles of the

human ovary there are relatively few macrophages but numbers increase dramatically in the developing follicle and immediately prior to ovulation. Similarly, quantitative analysis of immune cells such as macrophages in ovarian follicles from cyclic cows (Nakamura et al., 1987), cyclic rats (Gaytan et al., 1991; Brannstrom et al., 1993), and immature rats (Fukumatzu et al., 1992) indicated that a significant number of white blood cells are present in healthy antral and mature follicles. It is suggested that the resident ovarian white blood cells may constitute potential in situ regulation in the ovary (Bagavandoss et al., 1988; Harrison and Campbell, 1988; Adashi et al., 1989).

Corpus Luteum

The migration of macrophages, lymphocytes, monocytes, and NK cells into the regressing corpora lutea has been observed in many species of animals, including humans and cattle (Lei et al., 1991; Standaert et al., 1991; Wang et al., 1992a). Lymphocytic infiltration in the cyclic bovine corpus luteum was reported (Lobel and Levy, 1968) and the migration of eosinophils occurred before either functional or structural luteal regression in sheep (Murdoch, 1987). This infiltration did not occur to the same extent when compared pregnant and cyclic animals. Macrophages were identified in mouse corpora lutea (Kirsch et al., 1981) at the time when the luteal cells were secreting large amounts

of progesterone and there was no evidence of luteolysis which would affect steroid secretion. Similarly, Standaert et al., (1991) found that macrophages are the predominant blood cell type present in luteal tissue during specific stages of the porcine corpus luteum. Other studies have reported the presence of white blood cells in rabbit corpora lutea (Bagavandoss et al., 1990), demonstrating that the number of macrophages increased greatly with the regression of the corpus luteum. Lei et al., (1991) found an increasing number of macrophages in the human corpus luteum from early to late luteal phase, suggesting that macrophages could play dual function; i.e., luteotropic in early and mid-luteal phases and luteolytic in late luteal phase. These authors also determined that the number of luteal fibroblasts decreased in maximally functioning corpora lutea and then greatly increased in regressing corpora lutea. In summary, various blood leukocytes, most significantly macrophages, lymphocytes, and eosinophils, differentially migrate into specific structures of the ovary at specific stages of the estrous cycle of most mammals. These findings suggest that white blood cells may be involved in some reproductive events such as ovulation, differentiation of follicular tissue during luteinization, and luteal regression.

Presence and Action of Cytokines in the Ovary

In the same way white blood cells can be detected in

the ovary, cytokines (mostly IL-1, IFNs, and TNF) can be found in the ovary. The presence of cytokines in different parts of the ovary created the need to identify the functions of these factors in relationship to the cells where they were found and their role in reproduction.

Cytokines in the Follicle

Interleukins. Human follicular fluid contains considerable levels (1.58 fmol/0.1 ml) of IL-1 with a significant positive correlation between follicular fluid IL-1 and progesterone levels (Barak et al., 1992a). On the contrary, Fukuoka et al., (1989) reported that IL-1 markedly inhibited progesterone secretion by immature porcine granulosa cells, and the responsiveness of the cells in terms of cell growth and basal progesterone secretion were greater in immature granulosa cells than in differentiated or mature cells, suggesting that granulosa cells lose their response to IL-1 as they differentiate to luteal cells. Fukuoka et al., (1992) found that IL-1α, IL-1ß, and IL-2 had no significant effect on human chorionic gonadotropic (hCG)-stimulated progesterone secretion in luteinized human granulosa cells. In cultured rat granulosa cells, IL-6 also inhibits FSH-induced progesterone production (Gorospe et al., 1992). IL-1 did not affect basal or LH-stimulated progesterone secretion by human luteal cells (Barak et al., 1992a) as in previous studies in porcine (Fukuoka et al., 1989) and human granulosa cells (Wang et al., 1991).

Barak et al., (1992b) found that IL-1 inhibited (hCG)-stimulated aromatase activity and estradiol production by human granulosa cells. Similarly, IL-1α inhibited follicle-stimulating hormone (FSH)-induced estradiol secretion by human granulosa cells (Yasuda et al., 1990; Fukuoka et al., 1992), suggesting a role for this cytokine as a local antiluteolytic agent since IL-1 also inhibits morphological luteinization induced by LH (Fukuoka et al., 1989). IL-2 had no effect on estradiol secretion (Fukuoka et al., 1992). There are no previous studies reported that have measured an IL-6 effect on estradiol production by granulosa cells.

IL-1 inhibited FSH-stimulated cyclic-adenosine monophosphate (cAMP) generation by 80 to 90%, but IL-1 did not affect hCG-stimulated cAMP generation in cultured human luteinized granulosa cells (Fukuoka et al., 1992). In contrast, Sjögren et al., (1991) reported that IL-1 alone did not significantly increase cAMP accumulation compared to the control, but IL-1 at a concentration of 50 IU/ml enhanced LH-induced cAMP release by human granulosa cells. Similar results of enhanced LH-stimulated cAMP by porcine granulosa cells were found by Yasuda et al., (1990). IL-1 also shows the ability to decrease androstenedione production by thecal cells (Hurwitz et al., 1991a), and this effect may be due to an inhibition of steroidogenic transformations catalyzed by 17α-hydroxylase/17-20 lyase.

IL-1 may also play an important part in the process of follicular rupture at ovulation; it may stimulate PGE_2

production by monocytes and in turn its own production is stimulated by leukotrienes (Dinarello, 1991). It may be, therefore, that IL-1 directly modulates local weakening of the follicular wall, or is an indirect marker of inflammatory activity. After ovulation the follicular wall undergoes a reparative process. IL-1 stimulates fibroblast proliferation, collagen deposition and fibrinogen formation (Kampschmidt, 1984; Dinarello, 1991). In summary, IL-1 is a potent inhibitor of FSH-induced differentiation of immature pig granulosa cells by inhibiting FSH-induction of estrogen and progesterone production, and potentiating LH-induced cAMP generation (Yasuda et al., 1990).

Interferons. Another prominent cytokine present in follicular fluid is IFN (Grasso et al., 1988). IFN-γ is produced locally in the human ovary, specifically by $CD8^+$ T cells in theca and interstitial tissue (Grasso and Muscettola, 1990). It has been found that the administration of IFN to women with normal menstrual cycles reduces their serum levels of progesterone in the luteal phase and serum estradiol levels in the follicular phase without affecting gonadotropin levels (Kauppita et al., 1982). When rat and human granulosa cells are exposed to IFN in vitro, an inhibition in the production of progesterone is observed (Gorospe et al., 1988; Wang et al., 1992a; Fukuoka et al., 1992). IFN-α and IFN-ß had a similar effect, but not as pronounced as IFN-γ. Wang et al.,

(1992a) reported that at a high concentration of IFN-γ (1000 U/ml) decreased both basal and hCG-stimulated progesterone production by human luteal cells. Treatment of rat granulosa cells with recombinant rat IFN-γ in the presence of FSH, significantly inhibited FSH-stimulated aromatase activity and progesterone production in a dose-dependent manner (Xiao and Findlay, 1991). This effect was not seen when FSH was not present in the culture medium. It has also been reported that IFN-γ inhibits the ability of FSH to induce LH and hCG for receptor formation (Grasso and Muscettola, 1990; Fukuoka et al., 1992). Moreover, IFNs have an inhibitory effect on hCG- and FSH-stimulated cAMP generation in cultured rat (Xiao and Findlay, 1991) and human (Fukuoka et al., 1992) granulosa cells. Even though IFNs have inhibitory effects on progesterone secretion, it did not cause any changes in the human granulosa cell numbers or viability (Wang et al., 1992b). Therefore, IFNs are potent inhibitors of progesterone and estradiol production, and hCG- and FSH-stimulated cAMP generation by rat and human granulosa cells.

Tumor Necrosis Factor. Concentrations of TNF in bovine follicular fluid from early midcycle and preovulatory follicles, increase with days of the estrous cycle having its maximal concentration the day of ovulation (Fukumatzu et al., 1992). One day after of ovulation, while the corpus hemorragicum is still present, high concentrations of TNF can still be observed (Zolti et al., 1990). This and other

evidence that rat and cow granulosa cells (Roby and Terranova, 1988; Roby and Terranova, 1989), as well as thecal cells (Hurwitz et al., 1991a), contain and produce TNF indicate that TNF is probably a paracrine or autocrine mediator of theca and granulosa cell function. TNF mRNA expressed in rat ovary does not appear to due to the mRNA of white blood cells (Sancho-Tello et al., 1992). Immunoreactive TNF was localized in human ovary to the follicular and luteal compartment (Roby et al., 1990). Healthy antral follicles contained TNF in the antral layer of granulosa cells, and a greater amount of TNF was found in granulosa cells (24 hr of culture) from atretic follicles than in healthy follicles, indicating a relationship of TNF with follicular atresia (Roby et al., 1990). TNF immunolocalization was apparent throughout the entire granulosa cell layer of atretic follicles, and also it appears to be present in the follicular fluid surrounding the antral and pyknotic granulosa cells (Roby et al., 1990). Immunoreactive (Roby and Terranova, 1989) and bioactive TNF in corpora lutea (Bagavandoss et al., 1990) and granulosa cells (Zolti et al., 1990) indicates that ovarian cells and/or ovarian macrophages are sources of TNF production.

TNF inhibited rat (Emoto and Baird, 1988; Adashi et al., 1989), porcine (Veldhuis et al., 1991) and human (Fukuoka et al., 1992) granulosa cell progesterone production by about 80% compared to control. FSH increased basal progesterone production by rat granulosa cells, but

when TNF was present, FSH-stimulated progesterone production was significantly attenuated (Roby and Terranova, 1990). A similar inhibitory effect on progesterone production was observed in porcine granulosa cells exposed to TNF (Yasuda et al., 1990). On the other hand, other studies done with whole ovaries and(or) purified thecal cells, found that TNF stimulated rat thecal cell progesterone, 20α-dihydroprogesterone, 17α-dihydroprogesterone, and androstenedione production (Roby and Terranova, 1988b; Roby and Terranova, 1990; Roby et al., 1990). In contrast, TNF was found to inhibit LH-stimulated androgen production by purified thecal cells (Andreani et al., 1991). Reasons for these discrepancies between studies is unclear.

TNF may participate in regulation of follicular estrogen secretion, which could determine whether the follicle remains healthy or becomes atretic during its course of development (Roby and Terranova, 1990). In cultured rat granulosa cells, basal aromatase activity was very low in the absence or the presence of various concentrations of TNF, establishing that TNF cannot stimulate aromatase activity by itself (Emoto and Baird, 1988; Adashi et al., 1989). However, TNF inhibited FSH-induced aromatase activity in rat (Emoto and Baird, 1988; Adashi et al., 1989), pig (Yasuda et al., 1990), and human (Zolti et al., 1992) granulosa cells, having its maximum effect at 10 ng/ml of TNF added to the culture medium. In vivo, this inhibitory effect on aromatase activity in

granulosa cells would likely cause a decrease in estradiol concentrations in the follicular fluid. Adashi et al., (1989) reported that TNF treatment to granulosa cells resulted in a decrease in the ability of FSH to increase extracellular cAMP which indicates that TNF may affect proximal cAMP generation. However, TNF did not affect FSH-induced cAMP at sites distal to cAMP production (Roby and Terranova, 1990). Emoto and Baird, (1988) indicated that TNF had no effect on cell protein and cell viability in the presence or absence of FSH, suggesting a direct effect of TNF on the differentiated function of the granulosa cells. In summary, it is thought that TNF has participation in follicular atresia, since it stimulates thecal progesterone production (Roby and Terranova, 1990), inhibits LH-stimulated androgen production by theca (Andreani et al., 1991) and inhibits FSH-stimulated aromatase activity in granulosa cells (Emoto and Baird, 1988; Adashi et al., 1989).

Cytokines in the Corpus Luteum

Macrophages have been identified in the corpus luteum from adult rats, cattle, and humans, as these cells are involved in the phagocytosis of luteal cells during luteolysis (Lobel and Levy, 1968; Roby and Terranova, 1990; Wang et al., 1992). Thus, IL-1ß, IFNγ, and TNF as products of macrophages and other white blood cells, may play a role in the regulation of the corpus luteum. IL-1ß was found to

be a potent stimulator of PGE_2 and 6 ketoPGF$_{1\alpha}$ synthesis in bovine luteal cells (Nothnick and Pate, 1990), indicating a role of IL-1 in luteal PGE_2 production. These prostaglandins are necessary for normal maturation and lifespan of the corpus luteum. Analysis done in bovine luteal cells by Benyo and Pate (1992), showed that TNF and IL-1ß increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 6 ketoPGF$_{1\alpha}$ production in a dose dependent manner. Similar results were observed when granulosa-luteal cells from humans were cultured in the presence of hCG (Wang et al., 1992b). These results suggest that IL-1ß and/or TNF may play an important role in the maturation and lifespan of the corpus luteum after ovulation. No significant effect was observed in progesterone production, but a declining trend was observed when luteal cells were exposed to TNF (Benyo and Pate, 1992; Wang et al., 1992b).

Cytokines in Maternal Recognition of Pregnancy

In order to maintain a successful pregnancy in domestic mammals, the process of luteolysis has to be inhibited as well as the immune response from the mother to a foreign tissue. The conceptus is thought to be responsible for this signalling during the first few weeks of life. Conceptus signalling may be accomplished through the synthesis and secretion of an IFN-like polypeptide known as trophoblast protein-1 (Bazer et al., 1991; Hernandez-Ledezma et al.,

1992). This protein has been identified in sheep (oTP-1), bovine (bTP-1), and caprine conceptuses (Stewart et al., 1987; Stewart et al., 1989; Thatcher et al., 1989; Bazer et al., 1991; Mirando et al., 1991; Hernandez-Ledezma et al., 1992; Geisert et al., 1992). The conceptus IFN-like protein which is similar to bovine IFN-αII, has antiviral activity, and is effective against many viruses. Because of their structural resemblance, oTP-1 and IFN-αII compete for same receptor (Stewart et al., 1987; Stewart et al., 1989; Roberts, 1989; Roberts et al., 1989).

The mechanism by which oTP-1 prevent luteolysis is thought to be through the inhibition of endometrial $PGF_{2\alpha}$ release in response to estrogen and oxytocin triggering, and by suppressing inositol phosphate/diacylglycerol second messenger pathways for $PGF_{2\alpha}$ synthesis (Helmer et al., 1989; Bazer et al.,1991). Estradiol and oxytocin are necessary mediators of the release of $PGF_{2\alpha}$ (Bazer et al., 1991; Geisert et al., 1992) required for the initiation of luteolysis. Nonpregnant ewes that have been exposed to uterine infusion of oTP-1 between day 12 and day 18 have extended interestrous intervals through maintenance of the corpus luteum (Vallet et al., 1988). Another possible way that the inhibition of $PGF_{2\alpha}$ by bTP-1 or IFN-α is accomplished is by the stimulation of 2',5'-oligoadenylate synthetase activity (Mirando et al., 1991; Short et al., 1991). During maternal recognition of pregnancy ovarian follicular populations are altered in cattle (Thatcher et

al., 1989; Spicer and Geisert, 1992). Thus, cytokines may play a role in the suppression of follicular development during this early stage of pregnancy, by reducing the secretion of estradiol that could otherwise stimulate uterine secretion of prostaglandin $F_{2\alpha}$ (Thatcher et al., 1986). In support of these suggestions, administration of IFN-α to women, reduced estradiol and progesterone concentration in blood (Kauppita et al., 1982; Newton et al., 1990), and in vitro studies showed the same effect on luteinized human granulosa cells (Fukuoka et al., 1992). The exact mechanism of how cytokines work in maternal recognition remains to be elucidated, but more research is being done and many of the questions that we have currently, will be answered hopefully in the near future.

Disease Prevention and Future Application of Cytokines

The immune system is composed of many signalling pathways for communication between cells. It has been demonstrated that this communication is based on proteins known as cytokines, produced by the immune cells (e.g., macrophages) that will regulate the response of the body toward a foreign agent or inflammatory reaction. The regulatory mechanism involved in modulating the immune system is very delicate and complex at the same time. Cytokines can inhibit, enhance, or overlap each other's function, making it hard to observe the specific function

that each one has. With advancement of recombinant DNA and other technologies, we will better be able to obtain the required quantities of cytokines that can be used in the treatment of cancer, prevention of implanted tissue rejection, and improvement of production efficiency in the animal industry. For example, Babiuk et al. (1987) demonstrated that intra-muscular application of IFN-γ to cattle suffering bovine respiratory diseases (BRD) reduces the clinical signs of the disease. He also showed that treating with IFN-γ after infection was not as effective as when the treatment occurred prior to infection. IFN-γ can also be used in the prevention of intramammary infection (mastitis) by triggering the immune response. Because of the short half-life of IFN-γ, and the low therapeutic doses (10^5 U of IFN-γ per quarter) required for beneficial effects, cytokines have an advantage in the control of mastitis (Babiuk et al. 1990). Other cytokines such as IL-2 has been extensively used in the treatment of cancer (melanoma) in children and adult patients. TNF has been used in cancer treatment as well, but because of its cytotoxic effects, its use is just strictly for research. The administration of TNF-α to healthy animals (rats and cattle) induces cardiovascular collapse, multiorgan system failure, and death (Campos et al., 1991). Both passive immunization with antibodies to TNF-α and blockade of IL-1 receptors with IL-1 antagonist improves the survival in primates exposed to E.coli septic shock (Tracey et al. 1987; Fischer et al.,

1992).

The more open and practical use of cytokines in therapy will be seen in the near future, but before this, cytokines will have to be available in concentrations and quantities that doctors, patients, and producers can afford. More research should be done with cytokines in order to elucidate their practical use in human medicine and in the animal industry. Moreover, increased levels of cytokines, whether exogenously supplied or induced by disease endogenously, may have significant effects on reproductive functions, particularly ovarian steroidogenesis. Further research will be required to determine if cytokines mediate certain disease-induced reproductive failure in cattle.

CHAPTER III

EFFECTS OF CYTOKINES ON ESTRADIOL PRODUCTION BY BOVINE GRANULOSA CELLS IN VITRO: DEPENDENCE ON SIZE OF FOLLICLE

Introduction

Cytokines not only exert effects on the immune system but also exert effects on ovarian follicular cell steroidogenesis (Adashi, 1990a; Adashi, 1990b; Hurwitz et al., 1991; Barak et al., 1992b). Among these cytokines, the interleukins (ILs), interferons (IFNs), and tumor necrosis factor α (TNF-α) have been shown to have inhibitory effects on estrogen production by rat ovarian granulosa cells cultured in vitro (Gorospe et al., 1988; Adashi et al., 1989; Kasson and Gorospe, 1989). The sources of these cytokines within the ovary appear to be luteal (Roby and Terranova, 1989; Roby et al., 1990; Ji et al., 1991) and granulosa cells (Roby and Terranova, 1989; Roby et al., 1990) as well as white blood cells such as macrophages, eosinophils, leukocytes and T lymphocytes (Roby and Terranova, 1989; Nakamura et al., 1987; Cavender and Murdock, 1988; Hughes et al., 1990). Therefore, cytokines may act as local (paracrine or autocrine) regulators of ovarian function (Adashi, 1990a; Adashi, 1990b). Whether cytokines differentially affect granulosa cell functions in small and large follicles is unknown, but a recent study indicates that

granulosa cells from small and large follicles respond differently to conditioned medium from cultured splenocytes (a source of cytokines; Hughes et al., 1990). Also, reports on direct effects of cytokines on aromatase activity of bovine ovarian cells are scarce. Therefore, the objective of this study was to determine the effects of ILs, IFNs and TNF-α on in vitro cell aromatase activity of granulosa cells obtained from small and large bovine antral follicles.

Materials and Methods

Reagents and Hormones

Reagents used in the experiments were: Dubelcco's Modified Eagles Medium (DMEM), Ham's F12, insulin (bovine) and fetal calf serum (FCS) obtained from Sigma Chemical Co. (St. Louis, MO); ovine FSH (NIADDK-oFSH-17, FSH activity 20 x NIH-FSH-S1 U/mg; LH activity 0.04 x NIH-LH-S1 U/mg) obtained from the National Hormone and Pituitary Program (Baltimore, MD) exept for the IL-6 studies where oFSH-19 was used (USDA-oFSH-19-SIAFP-I-2, FSH activity 94 x NIH-FSH-S1 U/mg; LH activity 0.025 x NIH-LH-S1 U/mg); testosterone obtained from Steraloids Inc. (Wilton, NH); recombinant human IFN-α ($\geq$ 4 x 10^8 U/mg) and IL-6 ($\leq$ 0.1 ng endotoxin per μg) were obtained from R&D systems (Minneapolis, MN); recombinant human IL-1ß (< 0.1 ng endotoxin per mg), IL-2 (5 x 10^6 U/mg) and TNF-α (3.2 x 10^7 U/mg) obtained from Bachem (Torrance, CA); recombinant bovine IFN-β_2 and IFN-γ obtained from Genentech, Inc. (South San

Francisco, CA); and recombinant bovine trophoblast protein-1 (bTP-1; 1.5 x 10^7 U/mg; generously donated by Dr. R. Michael Roberts, University of Missouri, Columbia, MO).

Cell Culture

Ovaries were obtained from Quality Meats slaughter house (Wellington, KS) from beef, dairy cows, and heifers after slaughter. Ovaries were placed in saline solution (0.15 M NaCl), transported to the laboratory on ice (< 120 min), washed three times in saline solution (0.15 M NaCl), and then rinsed in ethanol solution (70% ETOH:30% saline solution) for 30 seconds and followed by rinsing three times with saline solution. Follicles were separated into two groups based on surface diameter: small (1 to 5 mm; containing mainly undifferentiated granulosa cells) and large (≥ 8 mm; containing mainly differentiated granulosa cells) as previously described (Langhout et al., 1991). Granulosa cells were collected by aspiration using a needle (20 gauge, 38.1 mm) and syringe (plastic, 3 ml) and washed twice in serum-free medium via centrifugation (100 X g for 5 min). Medium was a 1:1 mixture of DMEM and Ham's F12 containing 0.12 mM gentamicin and 38.5 mM sodium bicarbonate. Number of viable cells (24 ± 2% and 33 ± 4% of total cells obtained from small and large follicles, respectively) were determined using the trypan blue exclusion method (Adashi et al., 1987). Between 1 and 3 x 10^5 cells in 30 to 80 μl of medium were added to Falcon multiwell plates (#3047; Becton Dickinson and Co.,

Oxnard, Ca.) containing 1 ml medium. Cultures were kept at 37°C in a 5% CO_2 atmosphere, and medium was changed every 24 h as described by Langhout et al., (1991). To obtain optimal attachment, cells were maintained in the presence of 10% FCS for the first 2 days of culture, unless stated otherwise. After the attachment period, granulosa cells were washed twice with 0.5 ml serum-free medium and incubations continued in serum-free medium with or without hormones. For studies evaluating the effect of hormones on aromatase activity, hormonal treatments were applied for 1 day (i.e., day 2 to 3 of culture), unless stated otherwise.

Determination of Granulosa Cell Number

Numbers of granulosa cell were determined at the termination of experiments using a Coulter counter (Model Zm; Coulter Electronics, Haileah, FL) as previously described by Baranao and Hammond, (1985) and Langhout et al.,(1991). Briefly, cells were exposed to 0.5 ml of trypsin (0.25% wt/vol) for 20 min at 25°C, then scraped from each well with a teflon policeman, diluted in 0.15 M NaCl and enumerated.

Cell Viability

Viability of granulosa cells were determined at the beginning of the culture period and after 3 days in culture by a trypan-blue exclusion method previously described (Adashi et al., 1987).

Estradiol RIA

Concentrations of estradiol-17ß in culture medium were determined by radioimmunoassay (RIA) as previously described (Spicer and Enright, 1991). The intra- and interassay CV were 12 and 20%, respectively. Cross-reactivity for estrone and estradiol-17α expressed relative to estradiol-17ß, were 1.3% and 3.4%, respectively, as previously described (Cox and Britt, 1982). The doses of testosterone and the other hormones used did not crossreact in the RIA.

Statistical Analyses

Experimental data are presented as the least squares means ± SE of measurements from quadruplicate culture wells from two or more experiments. Each experiment was performed two to four times with different pools of granulosa cells collected from 20 to 60 ovaries for each pool. Only two experiments were performed if results from each experiment were in agreement with each other. Treatment effects and interactions were assessed using GLM procedures of SAS (SAS, 1988). Main effects were treatment (e.g., dose) and experiment when data from more than one experiment were analyzed. Each well was a replicate and each experiment contained four replicates per treatment. When estradiol was expressed as ($pg/10^5$ cells/24 h), cell numbers at the termination of the experiment were used for this calculation. Specific differences in cell growth and aromatase activity

between treatments were determined using Fisher's Protected Least Significant Difference Procedure (Ott, 1977).

Results

Assessment of Functional Aromatase Activity

Functional aromatase activity was determined during a 24-h exposure of granulosa cells to testosterone (1 or 3 μg/ml) as an estradiol precursor. These doses were maximal for estradiol production after 24-h incubation (data not shown). Conversion of testosterone to estradiol was time dependent and increased linearly from 4 to 24 h of incubation (Figure 1) and proportional to the number of cells (0.5 to 12 x 10^5 cells/well) plated (data not shown). To optimize hormonally sensitive aromatase activity in culture, granulosa cells were obtained from small and large follicles, cultured for either 1 or 2 days in the presence of 10% FCS and then treated with insulin (5 μg/ml) and(or) FSH (200 ng/ml) for an additional 24 h in the presence of 3 μg/ml testosterone. In cells from small and large follicles neither insulin nor FSH affected estradiol production of cells cultured for only 1 day with 10% FCS (Figure 2). Estradiol production was greater ($P<.05$) in cells from large follicles than that of small follicles on Day 1-2 of culture. Also, estradiol production decreased between Day 1-2 and Day 2-3 in control cells from small and large follicles and in insulin-treated cells from large follicles

(Figure 2). Insulin alone increased ($P<.05$) estradiol production by cells from small follicles but not by cells from large follicles. However, FSH increased ($P<.05$) estradiol production in the presence of insulin in cells from both small and large follicles. Thus, cultures in which cells were exposed for 2 days with 10% FCS and 1 day with hormones (1 μg/ml insulin and 200 ng/ml FSH) and 1 μg/ml testosterone were selected as optimal conditions for further experiments to evaluate estradiol production.

Effect of IL-1ß, IL-2 and IL-6 on FSH-induced Granulosa Cell Estradiol Production

To determine the effect of IL-1ß, IL-2 and IL-6 on FSH-induced estradiol production by bovine granulosa cells, cells were obtained from small and large follicles, cultured for 48 h in 10% FCS, and then cultured in the presence of FSH (50 or 200 ng/ml), insulin (1 μg/ml) and testosterone (1 μg/ml) for additional 24 h to maximize aromatase activity. During these 24 h, treatment of IL-1ß (0, 10 and 100 ng/ml), IL-2 (0 and 100 ng/ml) and IL-6 (0, 0.1, 1.0, 10.0, 30.0, and 100.0 ng/ml) were added to the culture medium. In cultures of granulosa cells from large follicles, IL-1ß, IL-2, and IL-6 (0.1 to 10.0 ng/ml) had no significant effect on FSH-induced estradiol production (Figures 3 and 4), whereas 30 and 100 ng/ml of IL-6 inhibited FSH-induced estradiol production by 62 and 50%, respectively (Figure 4). The ID_{50} for IL-6 was approximately 25 ng/ml for cells from large follicles. In cultures of

granulosa cells from small follicles, 100 ng/ml of IL-1ß and IL-2 inhibited FSH-induced estradiol production by 31% and 45%, respectively while 10 ng/ml of IL-1ß had no significant effect (P>.05) (Figure 3). All doses of IL-6 inhibited (P<.05) FSH-induced estradiol production with maximal inhibition achieved (75% decrease) with ≥ 10 ng/ml (Figure 5). ID_{50} for IL-6 was approximately 1.0 ng/ml for cells from small follicles. IL-1ß had no effect on cell numbers (Table 1). IL-2 reduced cell numbers by 27% in cultures of cells from small follicles (Table 1), but had no effect on cell viability (Table 2). At 100 ng/ml, IL-6 decreased (P<.05) numbers of cells from large follicles, and 1 to 100 ng/ml of IL-6 reduced (P<.05) cell numbers by 25 to 55 % in cells from small follicles (Figure 6).

Effect of Interferons on FSH-induced Granulosa Cell Estradiol Production

In cultures of granulosa cells from large follicles, 10 and 100 U/ml of IFN-ß and 100 U/ml of IFN-γ had no effect (P>.05) on FSH-induced estradiol production (Figure 7). In contrast, 100 U/ml of IFN-ß and IFN-γ inhibited (P<.05) FSH-induced estradiol production by 47% and 71%, respectively, in cultures of granulosa cells from small follicles; 10 U/ml of IFN-ß had no significant effect (Figure 7). IFN-α at 10 and 100 U/ml inhibited (P<.05) FSH-induced estradiol production by 18% and 22%, respectively in cultures of granulosa cells from large follicles, and by 20% and 61%, respectively in cultures

TABLE 1
EFFECT OF CYTOKINES (24 h TREATMENT) ON NUMBERS OF GRANULOSA CELLS COLLECTED FROM SMALL (1-5mm) AND LARGE (≥ 8mm) FOLLICLES

		Cell number (% of control)	
Cytokine	Dose	Small Follicles[a]	Large Follicles[b]
IL-1ß	10	97.7 ± 4.7	102.1 ± 2.8
	100	100.9 ± 4.7	107.1 ± 2.8
IL-2	100	*73.1 ± 5.6	94.9 ± 3.7
TNF-α	10	94.8 ± 5.2	112.6 ± 4.5
	100	99.5 ± 5.2	99.4 ± 4.5
IFN-α	10	93.3 ± 4.9	82.3 ± 4.6
	100	*79.9 ± 4.9	91.5 ± 4.2
IFN-ß	10	94.8 ± 6.0	100.7 ± 5.9
	100	92.1 ± 6.0	98.1 ± 6.1
IFN-γ	100	98.8 ± 5.9	121.1 ± 13.8
bTP-1	10	120.0 ± 8.7	118.4 ± 5.3
	100	96.0 ± 8.7	114.3 ± 4.5

[a]Data are mean ± SE of 2 separate experiments for each cytokine; averaged across experiments, control cultures averaged 0.94 ± .05 x 10^5 cells/well. *P<.05 vs controls.
[b]Data are means ± SE of 3 separate experiments for each cytokine; averaged across experiments, control cultures averaged 0.20 ± .01 x 10^5 cell/well. *P<.05 vs controls.

of cells from small follicles (Figure 7). IFN-ß and IFN-γ had no effect on cell numbers (Table 1). IFN-α (100 U/ml) reduced cell numbers by 20% in cultures of cells from small follicles only, but had no effect on cell viability (Table 2).

Bovine trophoblast protein-1 (bTP-1), which is an interferon-like protein produced by the conceptus, was also tested and found to have no effect ($P>.05$) on FSH-induced estradiol production by cultures of cells from large follicles (Figure 8). However, 100 U/ml bTP-1 inhibited ($P<.05$) FSH-induced estradiol production by cells from small follicles by 28% whereas 10 U/ml was without effect (Figure 8).

Effect of TNF-α on FSH-induced Granulosa Cell Estradiol Production

TNF-α had no effect ($P>.05$) on FSH-induced estradiol production by granulosa cells from large follicles when 10 and 100 ng/ml were applied (Figure 8). In contrast, 10 and 100 ng/ml of TNF-α inhibited ($P<.05$) FSH-induced estradiol production by 65% and 72%, respectively, in cultures of granulosa cells from small follicles (Figure 8). TNF-α had no effect on cell numbers (Table 1) or cell viability (Table 2).

TABLE 2

EFFECT OF CYTOKINES (24 h TREATMENT) ON VIABILITY OF GRANULOSA CELLS FROM SMALL FOLLICLES

TREATMENT	CELL VIABILITY (%)[a]
CONTROL	95.2 ± 1.4
IL-2	97.6 ± 1.4
TNF-α	96.8 ± 1.3
IFN-α	97.6 ± 1.4
IFN-ß	98.5 ± 1.5
IFN-γ	98.2 ± 1.5

[a]Data are means ± SE of 2 separate experiments for each cytokine.

Discussion

The results from the present studies indicate that cytokines have little or no effect on FSH-induced estradiol production by granulosa cells collected from large bovine follicles. In contrast to large follicles, cytokines have potent inhibitory effects on FSH-induced estradiol production by granulosa cells from small bovine follicles.

IL-1ß decreased FSH-induced estradiol production by granulosa cells from small follicles by 30% in the present study. This finding is in general agreement with Gottschall et al., (1989) and Kasson and Gorospe, (1989) who observed that IL-ß and (or) IL-α decreased FSH-induced estradiol production by 20% to 80% in granulosa cells from rat ovaries. Yasuda et al., (1990) observed similar effects of IL-1 on cultured porcine granulosa cells. Barak et al., (1992a) found that IL-1 inhibited hCG-stimulated aromatase activity and estradiol production by human granulosa cells. In addition hCG-stimulated granulosa cell progesterone production was decreased by a specific IL-1 inhibitor (Barak et al., 1992a). No effect was observed of IL-1ß or IL-2 in FSH-induced estradiol production by granulosa cells from large bovine follicles in the present study. Reasons for the discrepancies are unclear but may be do to the species differences and (or) differentiative state of the granulosa cell used for cell culture. Alternatively, varying levels of endotoxin in the various preparations of cytokines may contribute to differences in results among studies. In vitro, IL-2 has also

been shown to inhibit hCG-induced progesterone production by human granulosa-luteal cells (Wang et al., 1991) but stimulate (Kasson and Gorospe, 1989) or have no effect (Gottschall et al., 1988) on FSH-induced progesterone production by rat granulosa cells. In the present study, IL-6 decreased FSH-induced estradiol production by granulosa cells from small and large follicles by 75 and 62%, respectively. This is in agreement with others who observed that IL-6 inhibited FSH-induced progesterone production by rat granulosa cells (Gorospe et al., 1992). To our knowledge, no previous study has evaluated the effect of IL-6 on granulosa cell estradiol production. Collectively, these results indicate that IL-1ß inhibits FSH-induced estrogen production by granulosa cells regardless of species, whereas IL-2 inhibition of aromatase activity may be species-dependent. At present, it is unclear if species differences exist relative to effect of IL-6. Our studies also indicate that granulosa cell responsiveness to cytokines is dependent on size of follicle. Similarly, previous studies have indicated that granulosa cells from small porcine follicles respond to conditioned medium from cultured splenocytes (a source of cytokines) differently than granulosa cells from large porcine follicles (Hughes et al., 1990). Why granulosa cells from large bovine follicles are less sensitive to ILs than cells from small follicles is unclear. Perhaps highly differentiated granulosa cells (large follicles) contain much fewer or no receptors for ILs than undifferentiated granulosa cells (small follicles). Further

research is needed to verify this suggestion.

The inhibitory effect of IL-6 of proliferation of granulosa cells has not been reported previously; this inhibitory effect of IL-6 was more pronounced in cultures of cells from small than large follicles and was observed using serum-free as well as serum-containing medium (data not shown). Yasuda et al., (1992) found that interferon inhibited porcine granulosa cell proliferation in a dose-dependent manner. In contrast, others observed that tumor necrosis factor-α (TNF-α) stimulated proliferatiion of human granulosa-luteal cells (Yan et al., 1993) and porcine granulosa cells (Fukuoka et al., 1992) in a dose-dependent manner. Fukuoka et al., (1992) also observed a stimulatory effect of IL-1 on proliferation of porcine granulosa cells whereas IL-2 was without effect. On the other hand, in the present study IL-2 caused a significant inhibitory effect (27%) on cell proliferation of bovine granulosa cells from small follicles. Others working with rat granulosa cells observed no effect of TNF-α on cell proliferation (Darbon et al., 1989). Reasons for the discrepancies among studies on the effect of cytokines on granulosa cell proliferation is unclear, but may be due to the specific cytokine tested, species differences, and(or) differentiative state of the granulosa cell used for cell culture. Furthermore, because 1 and 100 ng/ml of IL-6 reduced cell number by 26 and 55%, respectively, and estradiol production was corrected for cell numbers, the inhibitory effect of IL-6 on estradiol production was not soley due to

decreased cell numbers. Whether viability of cells is affected by IL-6 was not determined in the present study and may account for some of the inhibitory effects of IL-6. This possibility will require additional experimentation.

In agreement with a previous study using rat granulosa cells (Gorospe et al., 1988), we observed an inhibitory (71% decrease) effect of IFN-γ on FSH-induced estradiol production by bovine granulosa cells. IFN-γ has also been shown to inhibit progesterone production by cultured bovine luteal cells (Fairchild and Pate, 1991). In addition, we observed significant inhibitory effects (20% to 61% decrease) of IFN-α on FSH-induced estradiol production by granulosa cells from small and large follicles. This is in contrast with other studies that showed no effect of IFN-α on FSH-induced estradiol production in rat granulosa cells (Gorospe et al., 1988; Adashi et al., 1989). Reasons for these differences are unknown but are likely due to species differences.

The present study also showed that IFN-ß and bTP-1 can decrease FSH-induced estradiol production by 47% and 28%, respectively in cultured bovine granulosa cells. Previous studies have not evaluated the effect of these two interferon-like cytokines on aromatase activity of granulosa cells. It was recently reported that estradiol concentrations in follicular fluid are significantly decreased in large ($\geq$ 8 mm) follicles on day 15 and 18 of pregnancy in cattle (Spicer and Geisert, 1992), suggesting that the presence of a developing conceptus early in pregnancy may inhibit follicular estradiol

production. Thus, we hypothesize that interferons, produced by the conceptus and(or) the conceptus-stimulated uterus, may promote luteal function in early pregnant cows by inhibiting follicular estrogen production, which is known to be involved in luteolysis of ruminant species (Niswender and Nett, 1988; Spicer and Geisert, 1992).

TNF-α decreased FSH-induced estradiol production by granulosa cells from small follicles by 72% in the present study. This is in agreement with others which found a potent (70%) inhibitory effect of TNF-α on estradiol production by rat granulosa cells (Adashi et al., 1989; Emoto and Baird, 1988). It has been found that TNF-α decreases progesterone production by rat granulosa cells (Darbon et al., 1989; Adashi et al., 1990; Roby and Terranova, 1990) and by bovine luteal cells (Benyo and Pate, 1992) in vitro. Moreover, it has been demonstrated that TNF-α does not affect cell numbers or viability (present study; Gorospe et al., 1988; Adashi et al., 1989) giving then, the possibility of another mechanism of action. TNF-α and IL-1 appear to act at the site of cAMP generation, inhibiting FSH-induced cAMP formation (Gottschall et al., 1988; Adashi et al., 1989; Darbon et al., 1989).

Similar to other studies with porcine granulosa cells (Haney and Schomberg, 1981; Stoklosowa et al., 1982), we observed a decrease in estradiol production with increased duration of culture. This decrease in estradiol production was more pronounced in cells from large follicles. As previously demonstrated (Haney and Schomberg, 1981; Anderson

et al., 1979), granulosa cells from large follicles initially had more aromatase activity than did cells from small follicles. In agreement with previous findings using porcine granulosa cells (May and Schomberg, 1981; Maruo et al., 1988), we observed an increase in FSH-induced estradiol production by bovine granulosa cells treated with insulin.

The cellular source of ovarian cytokines is uncertain, but may include blood-borne lymphocytes, leukocytes, macrophages and other immune cells (Nakamura et al., 1987; Cavender and Murdoch, 1988). Previous studies have shown that these cells infiltrate the follicular theca shortly after the LH surge (Nakamura et al., 1987; Cavender and Murdoch, 1988), that rat ovaries contain TNF-α mRNA (Sancho-Tello et al., 1992) and IL-1ß mRNA (Hurwitz et al., 1991), that human follicular fluid contains macrophages (Loukides et al., 1990), that bovine (Zolti et al., 1990) and human (Zolti et al., 1992) follicular fluid contains TNF-like activity, and that human follicular fluid contains measurable IL-6 levels (Buyalos et al., 1992). The levels of TNF-α in bovine follicular fluid (5 to 10 U/ml; Zolti et al., 1990) and IL-6 in human follicles (Buyalos et al., 1992) are similar to the lower effective dose of TNF-α and IL-6 tested in the present study. Alternatively, granulosa cells and luteal cells may produce cytokines, since TNF-α has been immunocytochemically localized within human (Roby and Terranova, 1990), rat (Roby and Terranova, 1989), and bovine (Roby and Terranova, 1989) granulosa cells, and bovine corpora lutea contain TNF-α mRNA

(Ji et al., 1991). In addition, rat granulosa cells secrete IL-6 when cultured in vitro (Gorospe et al., 1992). Thus, cytokines may act as autocrine or paracrine regulators of ovarian follicular function. Whether the effects of cytokines on estradiol production in the present study and in previous ones are due to the direct action of cytokines on the granulosa cells or due to the indirect effects via production of additional cytokines or factors by granulosa cells is unknown. Such possibilities will require further study. In addition, studies are required to elucidate the function of cytokines in the reproductive process in vivo.

In conclusion, we suggest that granulosa cells of small antral follicles are much more sensitive to the inhibitory effects of cytokines on aromatase activity and cell proliferation than cells from large antral follicles in cattle. This may provide an autocrine or paracrine mechanism whereby higher TNF-α activity in large follicles (Zolti et al., 1990) and a greater number of immune cells within large follicles (Nakamura et al., 1987) and regressing corpora lutea (Lei et al., 1991; Standaert et al., 1991) may inhibit estradiol production (i.e., differentiation) by smaller follicles, thus avoiding premature differentiation of future ovulatory follicles. During pregnancy, this inhibition of follicular estradiol production by cytokines may help maintain luteal function.

Figure 1. Effect of incubation time on insulin-stimulated conversion of testosterone into estradiol. Cells from small follicles were cultured for 2 days in the presence of 10% FCS. During the last 4, 8 or 24 h of culture, serum free medium containing 3 μg/ml testosterone was added concomitantly with 5 μg/ml insulin. Estradiol concentrations in medium and cell numbers were determined. Values are means of quadruplicate culture wells from two separate experiments. Pooled SE = 3 pg/10^5 cells.

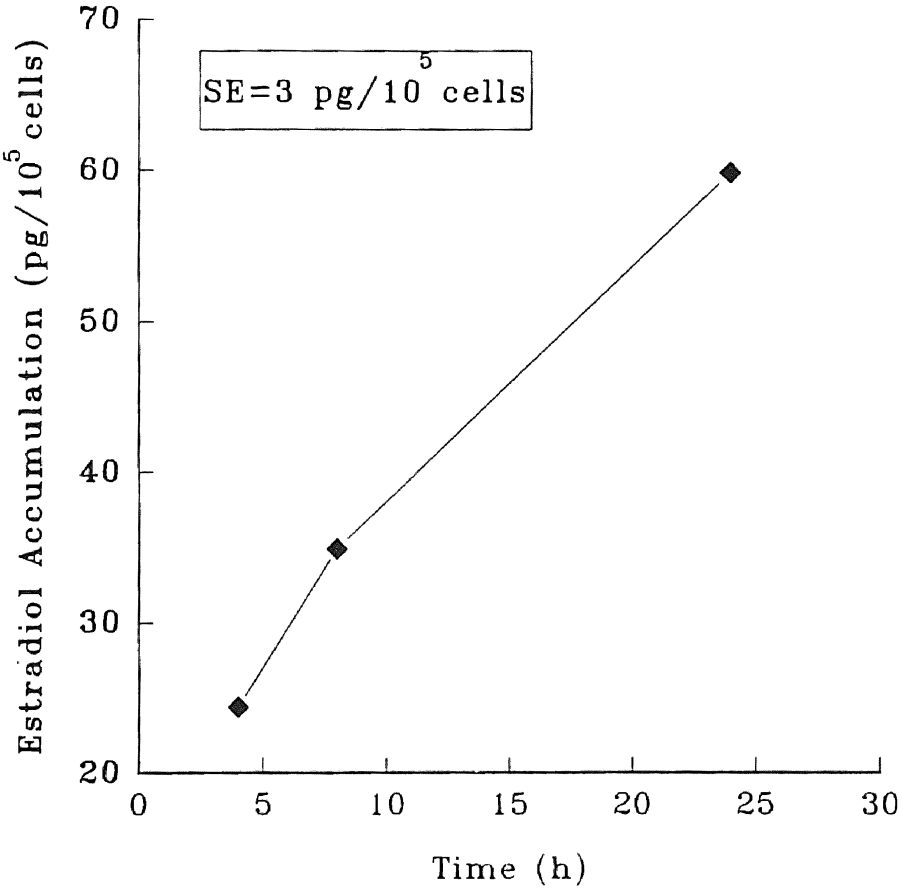

70
60
50
40
30
20
0
5
10
15
20
25
30
Estradiol Accumulation (pg/10^5 cells)
Time (h)
SE=3 pg/10^5 cells

Figure 2. Effects of insulin and(or) FSH on estradiol production by granulosa cells from large (Panel A) or small (Panel B) follicles. Granulosa cells were cultured for either 1 d or 2 d in the presence of 10% FCS and then treated with insulin (5 μg/ml) and(or) FSH (200 ng/ml) for an additional 24 h in the presence of 3 μg/ml testosterone. Values are least squares means of quadruplicate culture wells from two separate experiments. *, Mean differs (P<.05) from Day 2 value. **, Mean differs from control value. Pooled SE=8 and 10 pg/10^5 cells/24 h for Panels A and B, respectively.

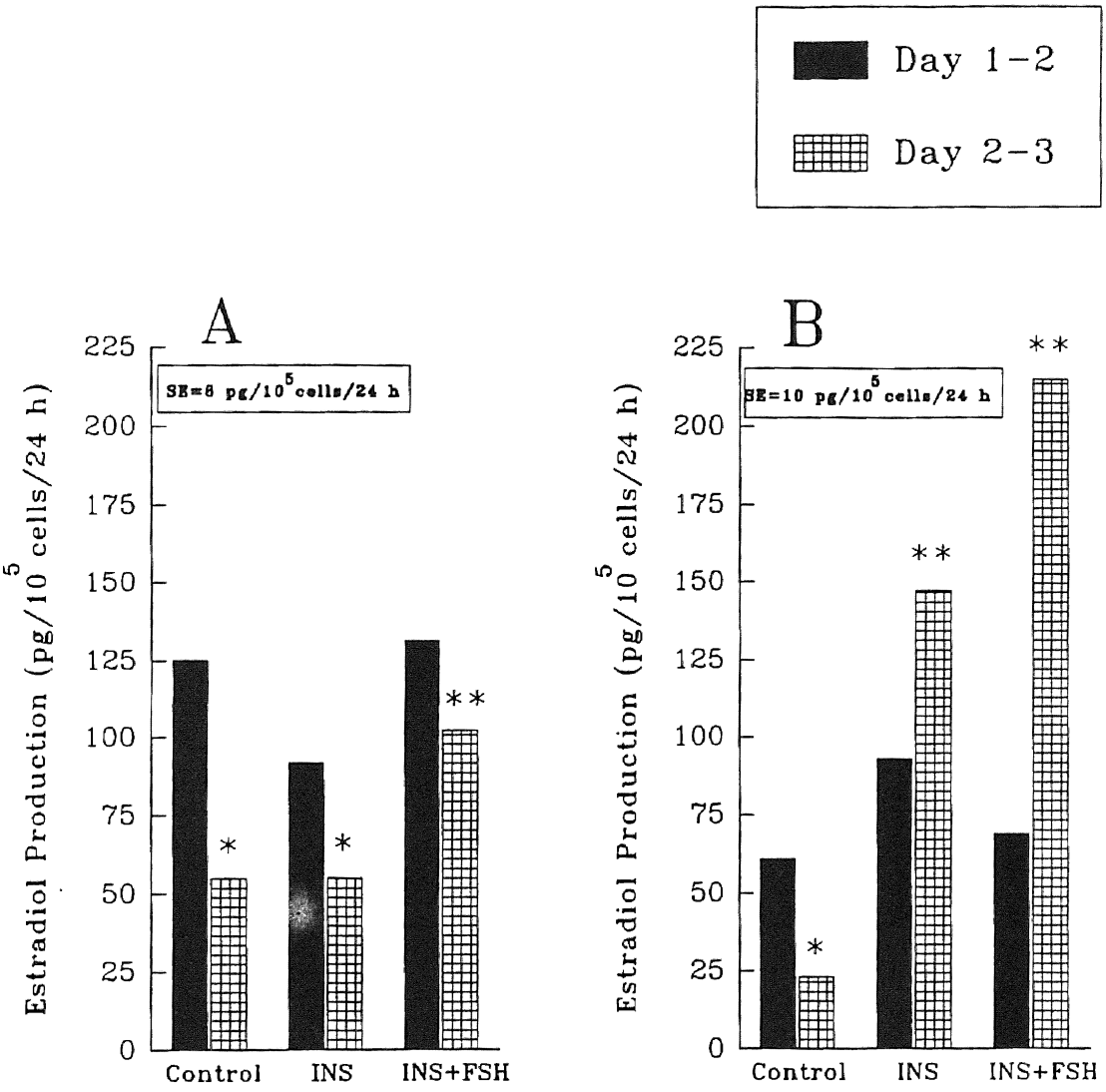

Day 1-2
Day 2-3
A
SE=8 pg/10^5 cells/24 h
B
SE=10 pg/10^5 cells/24 h
Estradiol Production (pg/10^5 cells/24 h)
225
200
175
150
125
100
75
50
25
0
*
**
Control
INS
INS+FSH
Treatments

Figure 3. Dose-response of IL-1ß and IL-2 on estradiol production by granulosa cells collected from large (Panel A) and small (Panel B) follicles. Granulosa cells were cultured for 2 d in the presence of 10% FCS and then treated with 200 ng/ml FSH, 1 μg/ml insulin and 1 μg/ml testosterone with or without the various doses of cytokines for an additional 24 h. Values are means of quadruplicate culture wells from three separate experiments for Panel A and two separate experiments for Panel B, and are expressed as percentage of control cultures which averaged 89 ± 6 and 65 ± 5 pg/10^5 cells/24 h for Panels A and B, respectively. *, Mean differs ($P<.05$) from control value (0 ng/ml).

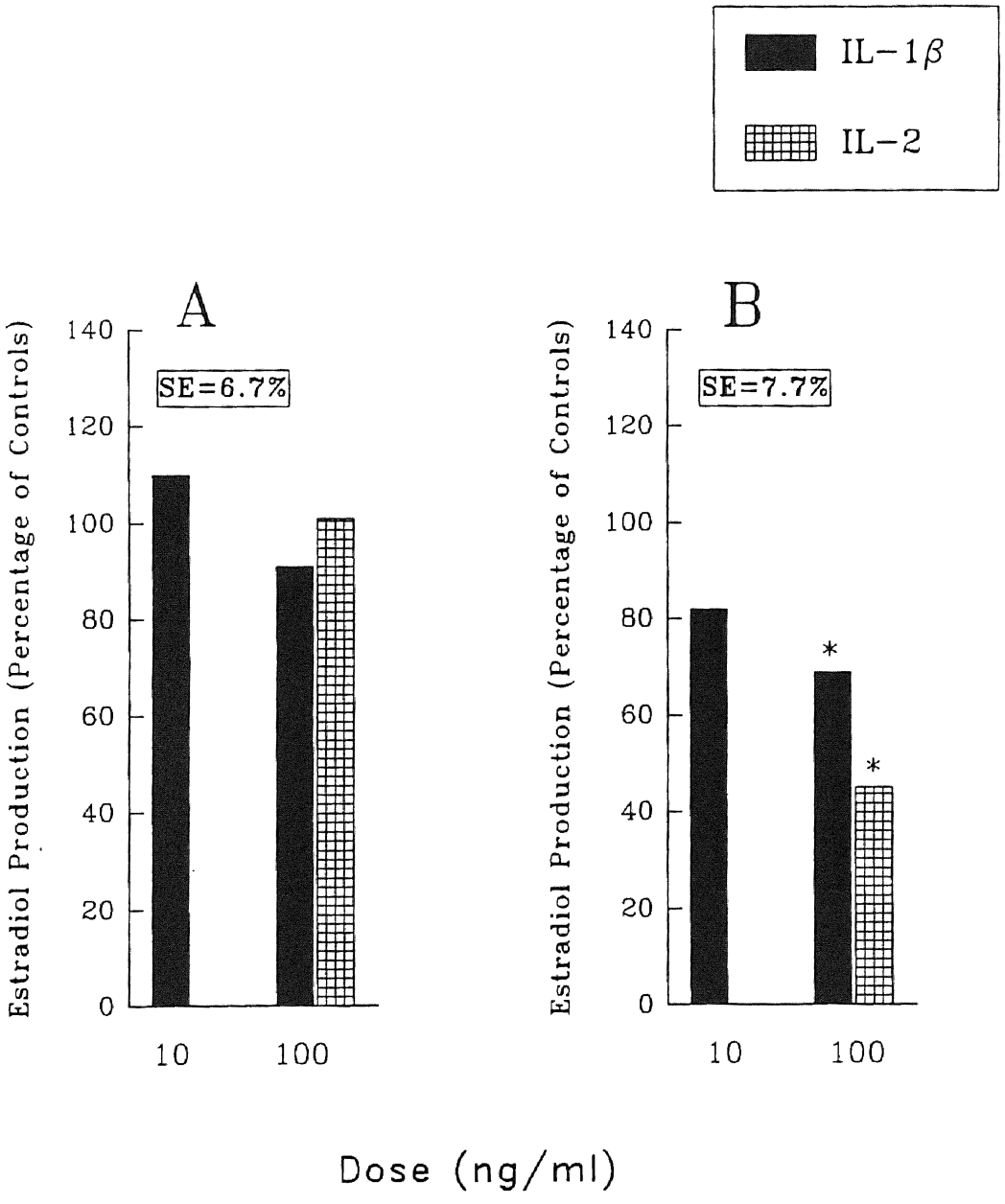

IL-1β
IL-2
A
SE=6.7%
B
SE=7.7%
Estradiol Production (Percentage of Controls)
140
120
100
80
60
40
20
0
10
100
*
*
Dose (ng/ml)

Figure 4. Effect of IL-6 on FSH-induced estradiol production by granulosa cells collected from large follicles. Granulosa cells were cultured for 2 days in the presence of 10% FCS as described in Materials and Methods, and then treated with 50 ng/ml FSH, 1 μg/ml insulin and 1 μg/ml testosterone with or without the various doses of cytokines for an additional 24 h. Values are means from three separate experiments, and are expressed as percentage of control cultures which averaged 119 ± 11 pg/10^5 cells/24 h. ^{a,b,c}Means with different superscripts differ ($P<.05$).

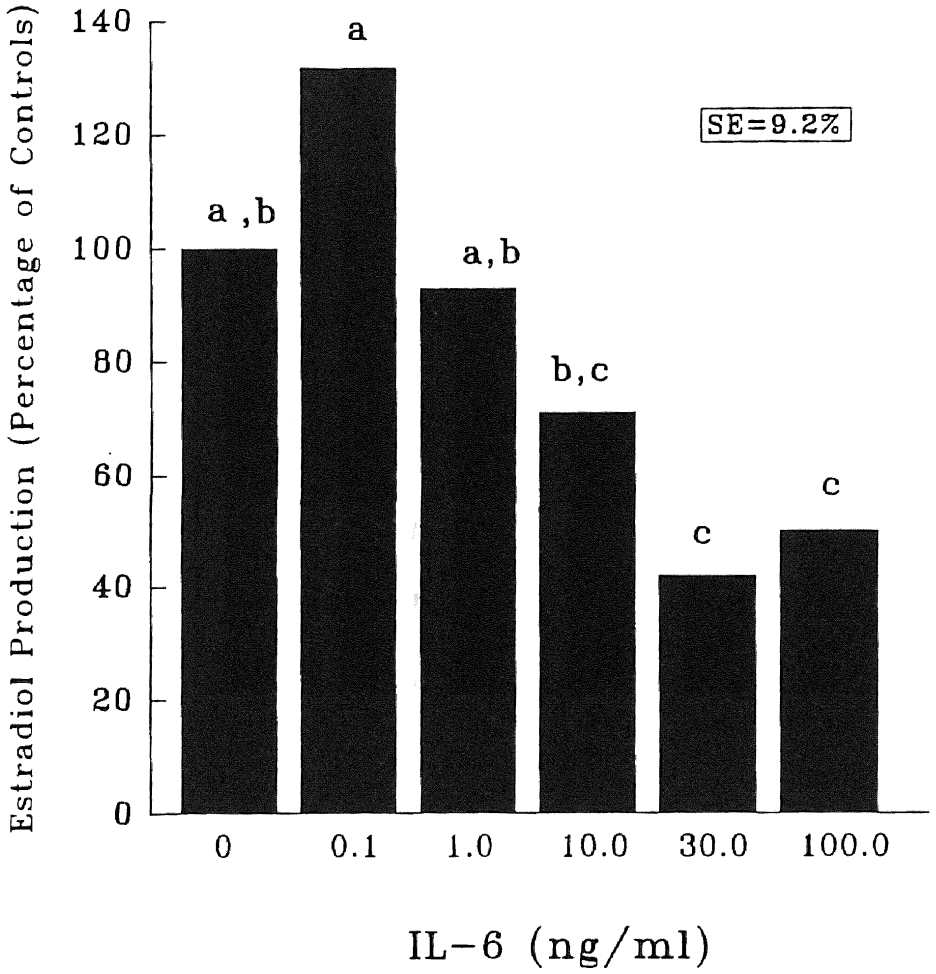

Estradiol Production (Percentage of Controls)
140
120
100
80
60
40
20
0
a
a ,b
a,b
b,c
c
c
SE=9.2%
0
0.1
1.0
10.0
30.0
100.0
IL-6 (ng/ml)

Figure 5. Effects of IL-6 on FSH-induced estradiol production by granulosa cells collected from small follicles. Cells were cultured as described in Figure 4. Values are means from three separate experiments, and are expressed as percentage of control cultures which averaged 90 ± 12 pg/10^5 cells/24 h. ^{a,b,c,d}Means with different superscript differ (P<.05).

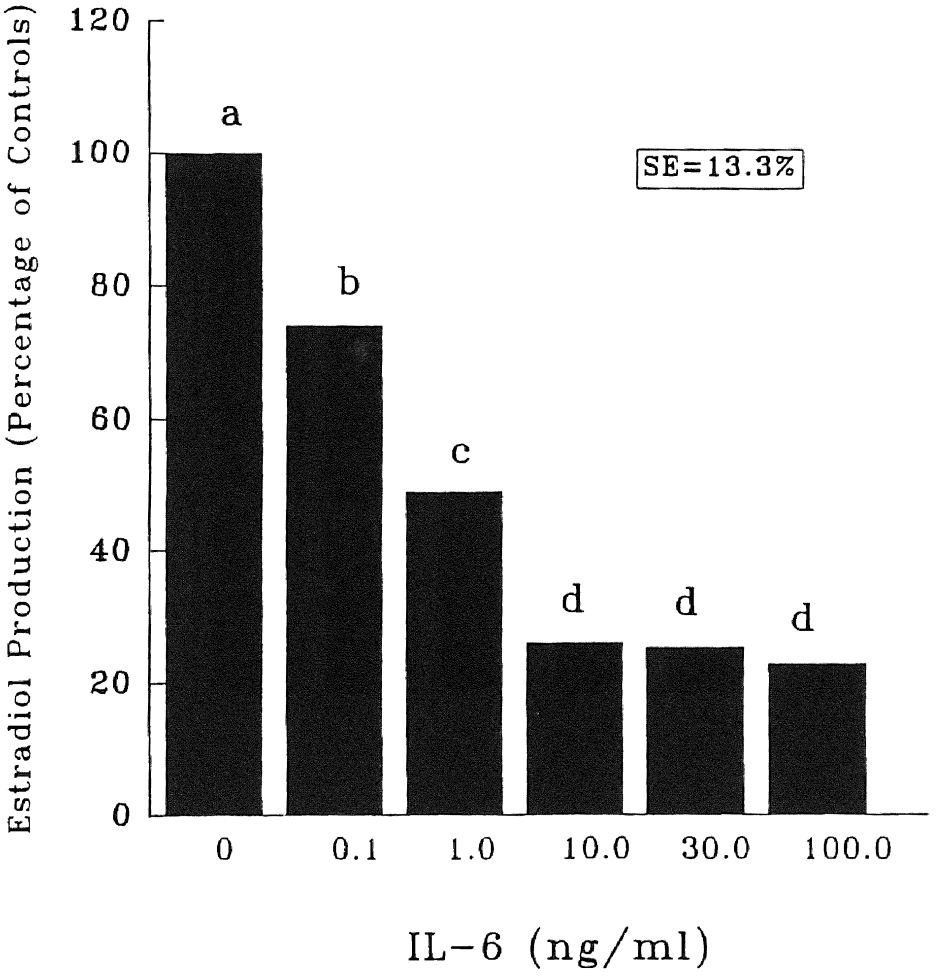

Estradiol Production (Percentage of Controls)
120
100
80
60
40
20
0
a
b
c
d
d
d
SE=13.3%
0
0.1
1.0
10.0
30.0
100.0
IL-6 (ng/ml)

Figure 6. Effect of IL-6 on in vitro proliferation of granulosa cells collected from large (———) and small (....) follicles. Cells were cultured as described in Figure 4. Values are means of three separate experiments, and are expressed as a percentage of control cultures which averaged 0.45 ± .03 and 1.25 ± .06 x 10^5 cell/well for large and small follicles, respectively.
[a,b,c,d]Within follicles size, means with different superscripts differ (P<.05).

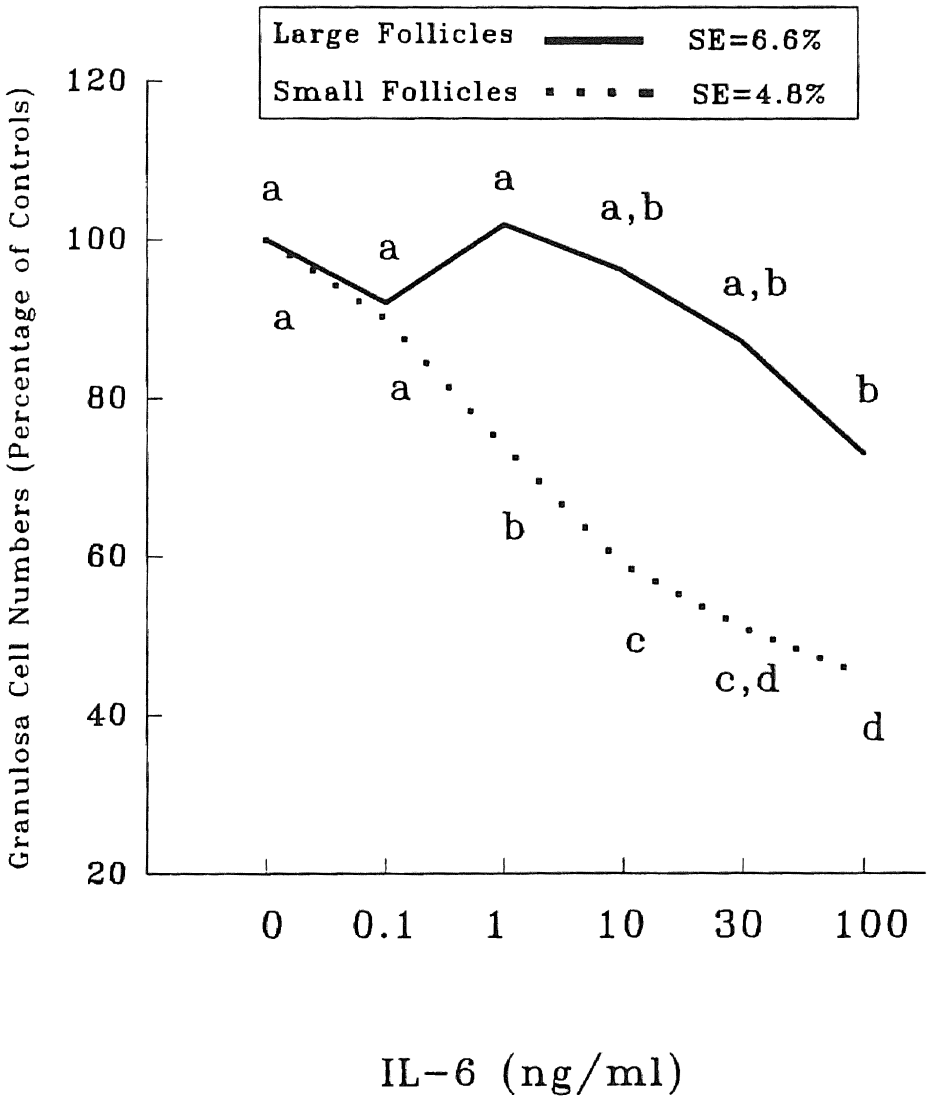

Large Follicles
SE=6.6%
Small Follicles
SE=4.8%
Granulosa Cell Numbers (Percentage of Controls)
120
100
80
60
40
20
a
a
a
a,b
a,b
b
a
b
c
c,d
d
0
0.1
1
10
30
100
IL-6 (ng/ml)

Figure 7. Dose-response of IFN-α IFN-ß and IFN-γ on estradiol production by granulosa cells collected from large (Panel A) and small (Panel B) follicles. Cells were cultured as described in legend for Figure 3. Values are means of quadruplicate culture wells from two separate experiments for Panel A and B, and are expressed as percentage of control cultures which averaged 103 ± 10 and 60 ± 4 pg/10^5 cell/24 h for Panels A and B, respectively. *, Mean differs (P<.05) from control value (0 U/ml).

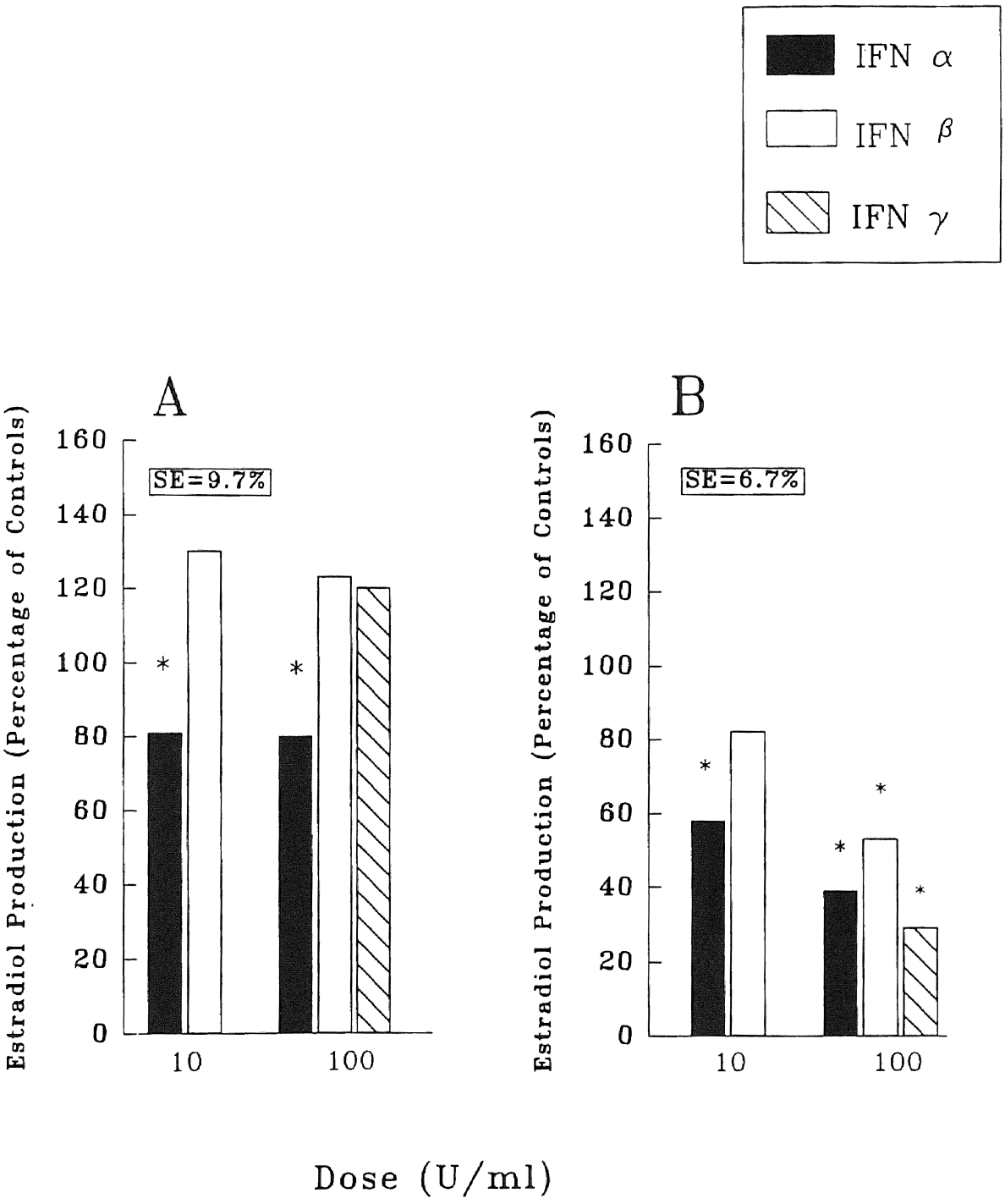
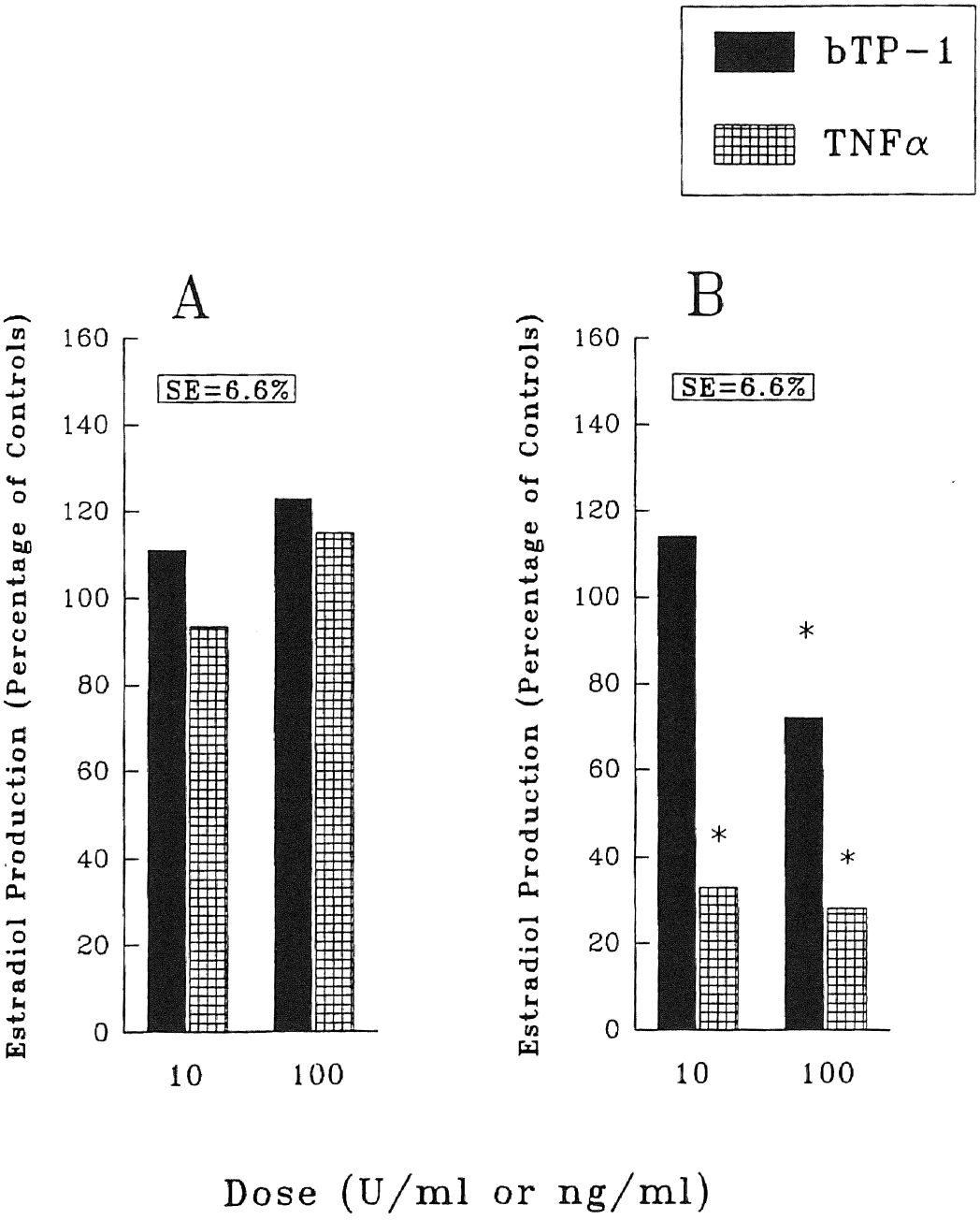

IFN α
IFN β
IFN γ
A
B
SE=9.7%
SE=6.7%
Estradiol Production (Percentage of Controls)
160
140
120
100
80
60
40
20
0
10
100
Dose (U/ml)

Figure 8. Effects of bovine trophoblast protein-1 (bTP--1 in U/ml) and TNF-α (ng/ml) on estradiol production by granulosa cells collected from large (Panel A) and small (Panel B) follicles. Cells were cultured as described in legend for Figure 3. Values are means of quadruplicate culture wells from three separate experiments for large follicles and two separate experiments for small follicles, and are expressed as percentage of control cultures which averaged 91 ± 6 and 61 ± 4 pg/10^5 cells/24 h for large and small follicles, respectively. *, Mean differs ($P<.05$) from control value (0 ng/ml or 0 U/ml dose).


bTP-1
TNFα
A
B
SE=6.6%
SE=6.6%
Estradiol Production (Percentage of Controls)
Estradiol Production (Percentage of Controls)
160
140
120
100
80
60
40
20
0
10
100
*
*
*
Dose (U/ml or ng/ml)

CHAPTER IV

GENERAL DISCUSSION

The immune system is regulated by glycoproteins produced by white blood cells such as macrophages, T lymphocytes, B lymphocytes, granulocytes and others. These glycoproteins are collectively called cytokines. They participate in inflammatory reactions and in the general immune response. The presence of white blood cells in porcine, rat, human and bovine ovaries during follicular and luteal phases has been reported by numerous studies. It is of great interest to observe the action of these white blood cells in the ovary as well as the possible effects that their products might have on folliculogenesis and steroidogenesis and therefore in reproduction.

Taking into consideration the presence of white blood cells in the ovary and their production of cytokines, as well as the production of some cytokines (IFNs and TNF-α) in the ovary itself, we might speculate about the possible ways by which cytokines may be affecting the reproductive system in vivo. Some authors suggested that cytokines may be involved in the ovulatory process, differentiation of follicular tissue during luteinization, luteal regression and follicular atresia. Some of the cytokines produced by white blood cells

mentioned in this report are interleukins (IL-1, IL-2, IL-6), interferons (IFN-α, β, γ) and tumor necrosis factor-α (TNF-α) which may have an autocrine and (or) paracrine function in the various ovarian cells. First of all, the ovulatory process is considered to be a modified inflammatory process that is thought to happened by a weakening of the follicular wall. IL-1 may be involved in the ovulatory process since IL-1 stimulates the production of prostaglandin E_2 (PGE_2) which has a luteotropic effect and it is necessary for the normal maturation of the corpora lutea. After ovulation the follicular wall undergoes a reparative process. IL-1 also exerts its action during this period through the stimulation of fibroblast proliferation, collagen deposition and fibrinogen formation that are known to be present in tissue during reparative processes after an inflammatory reaction.

It has been observed that these cytokines may have an inhibitory effect on estradiol production through the inhibition of aromatase activity which is necessary in the transformation of testosterone to estradiol by granulosa cells in conjunction with thecal cells when these cells have been cultured. It has been shown also that cytokines can inhibit progesterone production by human granulosa cells and the in vivo treatment of IFN-γ to women causes a decrease of serum levels of progesterone and estradiol. Follicular growth and therefore cell differentiation are directly affected by the presence of estradiol during the follicular phase. During early pregnancy, this growth has to be inhibited in order to

maintain the corpora lutea for further pregnancy. Maternal recognition of pregnancy in ruminants is also thought to involve an interferon-like protein, which inhibits the secretion of endometrial prostaglandin $F_2\alpha$ which acts as a luteolysin. It can be thought that this IFN-like protein may induce the production of other cytokines as well, that may affect the folliculogenesis during early pregnancy. IL-1 and IFNs coming from the conceptus may inhibit the growth of small follicles by the suppression of aromatase activity, androgen and estradiol production, as well as a possible decrease in granulosa cells in the follicle, causing low levels of estradiol inhibiting growth of possible ovulatory follicles allowing a better environment for the corpora lutea to maintain its full function.

Another normal process in the ovary is the atresia of the follicles that are not destine to ovulate. TNF may play an important role in follicular atresia. TNF been found in pyknotic granulosa cells undergoing atresia. Its specific role in this process is not known yet, but we can speculate that TNF is involved in the triggering of apoptosis and in the further degeneration of the follicles as well as in the regression of corpora lutea. Further experimentation is needed before any of these cytokines can be used in the treatment and(or) prevention of reproductive disorders such asovarian cysts and miscarriage during the recognition of pregnancy of ruminants.

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VITA 2

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