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EFFECTS OF ANDROGENS ON PROLIFERATION, VIABILITY, AND AROMATASE ACTIVITY OF BOVINE GRANULOSA CELLS IN VITRO

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EFFECTS OF ANDROGENS ON PROLIFERATION, VIABILITY, AND AROMATASE ACTIVITY OF BOVINE GRANULOSA CELLS *IN VITRO*

By

Christa M. A. Chilson

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ABSTRACT

EFFECTS OF ANDROGENS ON PROLIFERATION, VIABILITY, AND AROMATASE ACTIVITY OF BOVINE GRANULOSA CELLS *IN VITRO*

By

Christa M. A. Chilson

This study determined the effects of dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), 19-hydroxy-androstenedione (19-OH-A) and 19-hydroxy-testosterone (19-OH-T) on proliferation, viability, and aromatase activity of bovine granulosa cells (GCs) from first-wave dominant follicles. In Experiment I, GCs were cultured in media containing various doses of 19-OH-A, 19-OH-T, DHEA, DHT, or no androgen (NA) for 18 h. GC proliferation, viability, and estradiol production were then assessed. Treatment with androgens did not alter GC proliferation. However, viability of GCs was enhanced with 19-OH-A, 19-OH-T, or DHEA compared with NA or DHT treatment. GCs treated with 19-OH-A and 19-OH-T produced more estradiol and at a greater rate than those treated with DHEA. GCs treated with DHT or NA did not produce estradiol. In Experiment II, GCs were pre-treated for 6 h in media containing various amounts of 19-OH-A, 19-OH-T, DHEA, DHT, or NA. GCs were washed and cultured for a 12 h test period in media containing 19-OH-A or NA then aromatase activity was assessed. Pre-treatment with 19-OH-A or 19-OH-T enhanced aromatase activity compared with DHEA, DHT, or NA. In Experiment III, DHT diminished capacity of GCs to convert 19-OH-A to estradiol. In conclusion, type and amount of androgen altered GC viability, estradiol production, and aromatase activity implying that androgens have an important role in regulation of dominant follicle growth and atresia.

To my parents, Ronald and Carole Zielinski,

and my husband, Jim Chilson II,

for their love, support, and patience.

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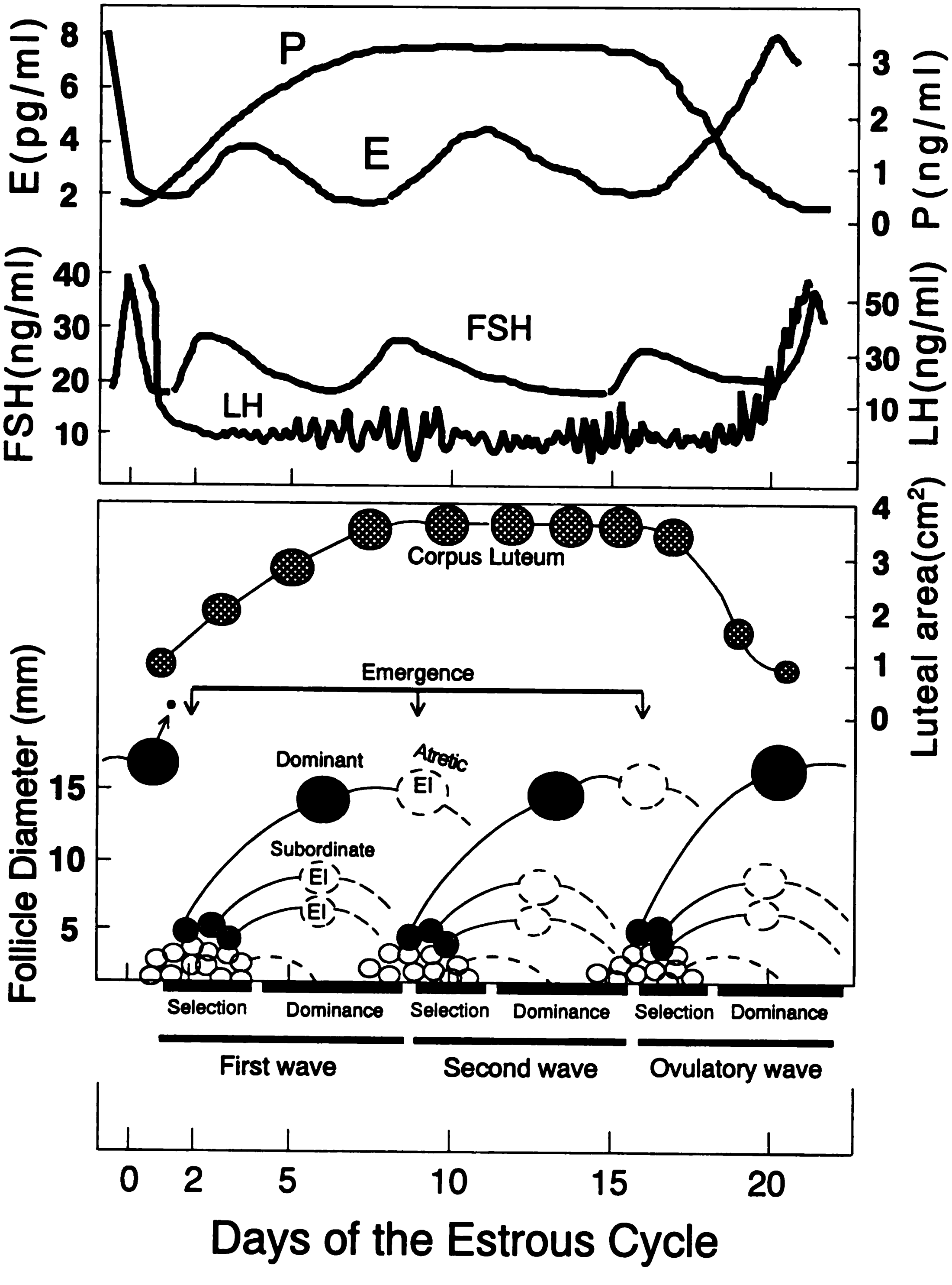
LIST OF ABBREVIATIONS

A	androstenedione
A_{405}	absorbance at 405 nanometers
ANOVA	analysis of variance
C	Celsius
cm	centimeter(s)
CO_2	carbon dioxide
DHEA	dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
E	estradiol-17β
E_1	Estrone
E_2	estradiol-17β
EA	estrogen-active
EDTA	ethylenediamine tetraacetic acid
EI	estrogen-inactive
fg	femtogram(s)
FSH	follicle-stimulating hormone
g	gravity
GC	granulosa cell(s)
h	hour(s)
LH	luteinizing hormone
M	molar
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mRNA	messenger ribonucleic acid
NA	no androgen
NaCL	sodium chloride
NaN_3	sodium azide
ng	nanogram(s)
nm	nanometer(s)
19-OH-A	19-hydroxy-androstenedione
19-OH-T	19-hydroxy-testosterone
P	progesterone
$P450_{arom}$	aromatase cytochrome P450
pg	picogram(s)
pH	hydrogen-ion concentration
RT	room temperature
SEM	standard error of the mean
SFM	serum-free medium

SFM-growth	Endothelial-SFM growth medium
SFM-plating	Endothelial-SFM plating medium
T	testosterone
t	time
μl	microliter(s)

Figure 1. The ovarian process of dominant follicle development in cattle.

Explanation provided in Introduction. (Figure from Jiménez-Krassel, 1997).


E (pg/ml)
8
6
4
2
P
E
P (ng/ml)
3
2
1
0
FSH(ng/ml)
40
30
20
10
FSH
LH
LH (ng/ml)
50
30
10
Luteal area(cm²)
4
3
2
1
0
Corpus Luteum
Emergence
Dominant
Atretic
El
Subordinate
El
El
Follicle Diameter (mm)
15
10
5
Selection
Dominance
Selection
Dominance
Selection
Dominance
First wave
Second wave
Ovulatory wave
0
2
5
10
15
20
Days of the Estrous Cycle

INTRODUCTION

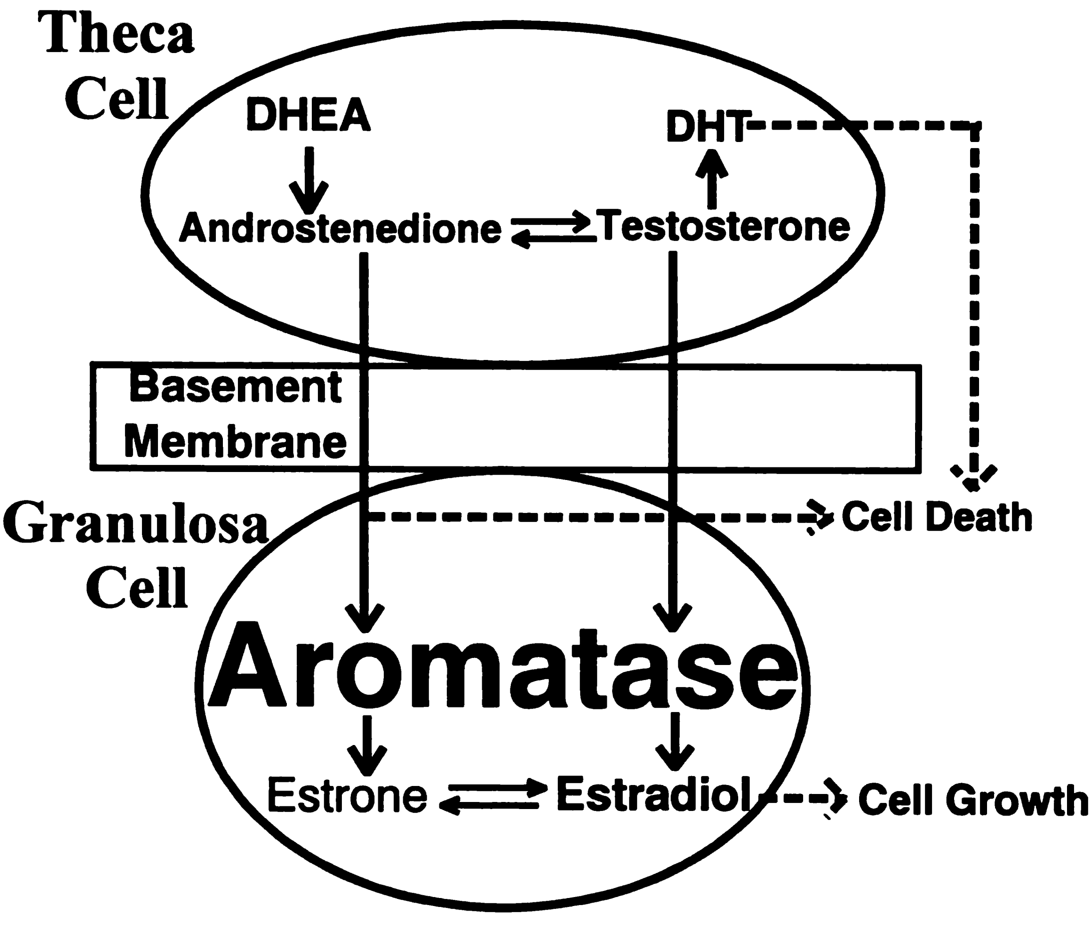
The bovine estrous cycle is approximately 21 days in length. Development and growth of ovarian follicles during this period occurs in two to three distinct patterns or "waves" with the final one ending in ovulation (Figure 1; Rajakoski, 1960; Matton *et al.*, 1981; Ireland and Roche, 1987; Fortune, 1993; Ginther *et al.*, 1996; Roche, 1996). Based on ultrasonographic studies, follicular waves begin on Days 2 and 11 of the estrous cycle in heifers exhibiting two waves and on Days 2, 9, and 16 in heifers exhibiting three waves (Figure 1; Savio *et al.*, 1988; Sirois and Fortune, 1988). During each wave a cohort of 3 to 6 follicles less than 5 mm in diameter simultaneously begin to grow (Sirois and Fortune, 1988; Bodensteiner *et al.*, 1996a). This event is referred to as emergence (Figure 1; Ginther *et al.*, 1996). One to 2 days after emergence, a follicle from the cohort grows larger than the others and becomes the dominant follicle (Fortune, 1993) while the subordinate follicles regress (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989). Besides using ultrasonographic analysis to characterize dominant follicle development, each wave of dominant follicle development consists of three theoretical growth phases: recruitment, selection, and dominance. During recruitment, a cohort of dormant, primordial ovarian follicles begins to grow. Out of this cohort, a follicle is "selected" to escape atresia and continue to grow. This selection process ends in development of a dominant follicle which continues to grow while the non-selected or subordinate follicles undergo atresia (apoptosis) and regress. No new follicles 5 mm or greater in diameter appear while the dominant follicle is growing. However, once the dominant follicle either starts to become atretic, as in the first two waves (Figure 1), or ovulates, as in the ovulatory wave (Figure 1), a new cohort of follicles is recruited and another wave begins (Goodman and Hodgen, 1983; Ireland and Roche, 1982; 1983a; 1983b). Dominant follicles can also be identified by intrafollicular concentrations of hormones. Dominant follicles have higher intrafollicular ratios of estradiol (E) to progesterone (P) concentrations and secrete more estradiol than all other follicles, thus they are classified as estrogen-active (EA) (Figure 1). Subordinate follicles contain more progesterone than estradiol in their follicular fluid, thus they are classified as estrogen-inactive (EI) (Figure 1; Ireland and Roche, 1982; 1983a; 1983b; Sunderland *et al.*, 1994). Understanding dominant follicle development (Figure 1) and its regulation are essential to increasing reproductive efficiency in cattle and improving methods to regulate the estrous cycle and increase fertility.

A transient increase in basal concentration of follicle-stimulating hormone (FSH) in serum precedes each follicular wave in heifers (Figure 1; Adams *et al.*, 1992; Sunderland *et al.*, 1994), therefore FSH may be the physiological "trigger" that activates the cyclic pattern of follicular growth. The decline of this transient increase in FSH is associated with the end of the selection process, growth of the dominant follicle, and atresia of the subordinate follicles (Roche, 1996; Ginther *et al.*, 1996). When FSH levels are low in serum, luteinizing hormone (LH) becomes important for dominant follicle growth (Figure 1). The dominant follicle gains LH receptors as it grows (Ireland and Roche, 1982; 1983a; 1983b; Xu *et al.*, 1995b; Bodensteiner *et al.*, 1996b) and depends on LH for survival in a hormonal environment that inhibits growth of subordinate follicles (Ginther *et al.*, 1996; Roche, 1996). However, within 3 to 7 days after a dominant follicle appears in waves 1 and 2, a decrease in the frequency of episodic LH secretion is linked to atresia of the dominant non-ovulatory follicle (Cupp *et al.*, 1995), whereas an increase in episodic LH secretion stimulates the dominant ovulatory follicle to ovulate (Figure1; Rahe *et al.*, 1980; Cupp *et al.*, 1995).

Although FSH and LH are the primary hormones that regulate ovarian follicle growth and function (Richards, 1980; Niswender and Nett, 1994; Jiménez-Krassel, 1997), numerous intraovarian factors including steroids, pituitary hormones, and growth factors interact with gonadotropins to regulate proliferation, viability and steroidogenesis of theca and granulosa cells, the two predominant cell types that comprise an ovarian follicle (Payne and Hellbaum, 1955; Schwarzel *et al.*, 1973; Louvet *et al.*, 1975; Scaramuzzi *et al.*, 1977; Hillier and Ross, 1979; Hillier *et al.*, 1979;1980a; Hillier, 1981; Farookhi, 1981; Terranova and Greenwald, 1981; Billig *et al.*, 1993; Luciano *et al.*, 1994; Evans *et al.*, 1997). However, the primary intrafollicular factor that modifies gonadotropin action is estradiol. Specifically, estradiol synergizes with gonadotropins to enhance number of gonadotropin receptor sites on granulosa and theca cells (Goldenberg *et al.*, 1972; Richards and Midgley, 1976; Richards *et al.*, 1976; Ireland and Roche, 1983a) and follicular growth (Goldenberg *et al.*, 1972; Hillier, 1981; Ireland, 1987; Fauser *et al.*, 1989) . In addition, estradiol synthesis (explained in Figure 3) is required for FSH-induction of its own receptor in granulosa cells (Tonetta and Ireland, 1984; Tonetta *et al.*, 1985). Estradiol production is associated with follicular growth (Hillier, 1981), and estradiol is anti-atretogenic (Payne and Hellbaum, 1955; Hillier, 1981; Billig *et al.*, 1993; Luciano *et al.*, 1994). Taken together, regulation of estradiol production is clearly a key step involved in follicular growth, differentiation, and atresia.

In cattle, estradiol is positively associated with selection, growth, and dominance

Figure 2. A hypothetical model showing how different types of androgens produced by a theca cell could regulate growth, viability and aromatase activity of a granulosa cell. Further explanation provided in Introduction. (Figure based on review of literature; Williams, 1940; Payne and Hellbaum, 1955; Goldenberg *et al.*, 1972; Lacroix *et al.*, 1974; Markis and Ryan, 1975; Fortune and Armstrong, 1977; Hillier, 1981; Hansel and Convey, 1983; Mc Natty *et al.*, 1984b; Tonetta and Ireland, 1984; Richards *et al.*, 1987; Payne *et al.*, 1992; Turzillo and Fortune, 1992; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997).


Theca Cell
DHEA
DHT
Androstenedione
Testosterone
Basement Membrane
Granulosa Cell
Cell Death
Aromatase
Estrone
Estradiol
Cell Growth

and negatively associated with atresia of ovarian follicles. Specifically, each wave of dominant follicle growth is characterized by an increase in estradiol production from those follicles escaping atresia. The dominant follicle produces considerably more estradiol than subordinate or atretic follicles (Figure 1; Padmanabhan *et al.*, 1984; Ireland and Roche, 1987; Fortune, 1994), implying that estradiol production has an important role in dominant follicle growth in the cow.

A variety of androgens are produced in theca cells of bovine follicles (Markis and Ryan, 1975; Fortune and Armstrong, 1977; Turzillo and Fortune, 1992) and cross the basement membrane and enter granulosa cells (Figure 2). Two different androgens, androstenedione and testosterone, are converted to estradiol by the aromatase enzyme system in granulosa cells (Figure 2; Lacroix *et al.*, 1974; Hansel and Convey, 1983; Richards *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). While androstenedione and testosterone are needed for estradiol production other androgens such as dihydrotestosterone (DHT), which is also produced in theca cells, inhibit estradiol production (Schwarzel *et al.*, 1973; Hillier and Ross, 1979; Hillier *et al.*, 1979; 1980a; 1980b) and induce atresia (Louvet *et al.*, 1975; Scaramuzzi *et al.*, 1977; Hillier and Ross, 1979; Hillier *et al.*, 1979; Farookhi, 1981; Hillier, 1981; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Billig *et al.*, 1993; Gore-Langton and Armstrong, 1994; Evans *et al.*, 1997). Since androgen receptors are in granulosa cells (Takeda *et al.*, 1990; Hild-Petito *et al.*, 1991; Horie *et al.*, 1992; Suzuki *et al.*, 1994; Tetsuka *et al.*, 1995; Garrett and Guthrie, 1996; Tetsuka and Hillier, 1996), androgens may be important regulators of estradiol production, and thus growth of a dominant follicle, not only by providing substrate for estradiol synthesis, but also by regulating aromatase activity and atresia of granulosa cells.

The overall hypothesis for my thesis research is that different types and amounts of androgens alter proliferation, viability, estradiol production, and aromatase activity *in vitro* of bovine granulosa cells isolated from the first-wave dominant follicle (Figure 2). The specific objective of my research was to determine if 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), or DHT affected proliferation, viability, and aromatase enzyme activity of bovine granulosa cells.

This thesis is in three parts: Chapter 1 reviews literature pertinent to synthesis of estrogens in granulosa cells from androgen substrates and the role of androgens in regulation of granulosa cell proliferation, viability, and estradiol production; Chapter 2 explains the development and validation of techniques used to test my hypothesis; and Chapter 3 presents results of the following three studies:

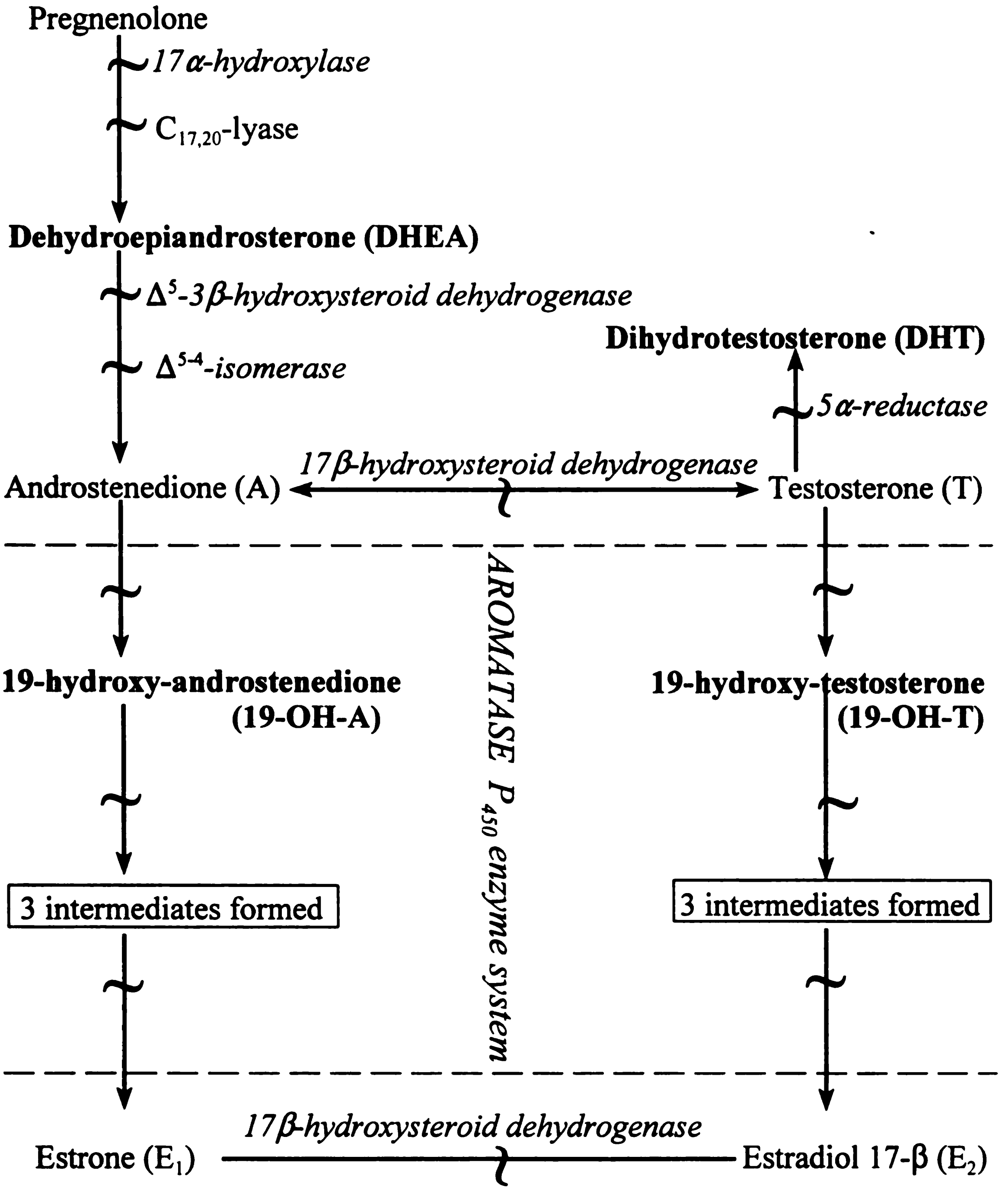
I) Effects of types and amounts of androgens on the proliferation, viability, and estradiol production of bovine granulosa cells from first-wave dominant follicles *in vitro*,

II) Effects of types and amounts of androgen on aromatase enzyme activity of bovine granulosa cells from first-wave dominant follicles *in vitro*, and

III) Effects of DHT on the production of estradiol by bovine granulosa cells *in vitro* (Chapter 3).

Figure 3. Estradiol synthesis in the ovary.

The metabolism of pregnenolone to estradiol in ovaries by the 5-ene-3β-hydroxy (or Δ^5) pathway involves several microsomal enzyme systems. The C_{21} side-chain cleavage P450 enzyme system, consisting of 17 α-hydroxylase and $C_{17,21}$-lyase, converts pregnenolone to dehydroepiandrosterone (DHEA). DHEA is then converted to androstenedione by the Δ^5 -3β-hydroxysteroid dehydrogenase: $\Delta^{5\text{-}4}$ - isomerase enzyme complex. The conversion of the C_{19} -steroids to estrogens by the aromatase P450 enzyme system is accomplished in a number of reactions with 19-hydroxy-androstenedione (19-OH-A) and 19-hydroxy-testosterone (19-OH-T) being two of the resulting intermediates. In bovine ovarian follicles, DHEA, androstenedione, testosterone, and DHT are synthesized from pregnenolone in theca cells (Lacroix *et al.*, 1974; Mc Natty *et al.*, 1984b; Turzillo and Fortune, 1992). In contrast, DHEA can be converted to androstenedione, testosterone, DHT, estrone, and estradiol by granulosa cells (Lacroix *et al.*, 1974; Mc Natty *et al.*, 1984b; Payne *et al.*, 1992; Turzillo and Fortune, 1992). Enzymes are in *italics* and "~" indicates where they act. Also note, aromatase P450 enzyme system participates in the reactions between the dashed lines. Steroids in **bold** were used in studies for this thesis (Chapter 3). Figure based on the following references: Hansel and Convey, 1983; Steinkampf *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997.


Pregnenolone
17α-hydroxylase
C17,20-lyase
Dehydroepiandrosterone (DHEA)
Δ5-3β-hydroxysteroid dehydrogenase
Δ5-4-isomerase
Dihydrotestosterone (DHT)
5α-reductase
17β-hydroxysteroid dehydrogenase
Androstenedione (A)
Testosterone (T)
AROMATASE P450 enzyme system
19-hydroxy-androstenedione (19-OH-A)
19-hydroxy-testosterone (19-OH-T)
3 intermediates formed
3 intermediates formed
17β-hydroxysteroid dehydrogenase
Estrone (E1)
Estradiol 17-β (E2)

Part I

CHAPTER 1

REVIEW OF LITERATURE

Synthesis of estrogens from androgen substrates

Androgens are a family of C_{19} (number of carbon atoms in their molecular structure) steroid hormones which are produced in the theca interna cells of ovarian follicles (Figure 2 and 3; Markis and Ryan, 1975; Fortune and Armstrong, 1977; Turzillo and Fortune, 1992). Synthesis of androgens by theca cells is primarily under the control of the pituitary gland hormone, luteinizing hormone (LH) (Collip *et al.*, 1933; Selye and Collip, 1933; Selye *et al.*, 1933; Papinicolaou and Falk, 1934; Price and Ortiz, 1944; Mc Natty *et al.*, 1984a). Bovine theca cells preferentially use the 5-ene-3β-hydroxy (or Δ^5) pathway to synthesize androgens from pregnenolone (Figure 3; Lacroix *et al.*, 1974; Fortune, 1986a, Fortune and Quirk, 1988; Gore-Langton and Armstrong, 1994). The conversion of pregnenolone to dehydroepiandrosterone (DHEA) by the C_{21} side-chain cleavage P450 enzyme system, which is the first step in the Δ^5 pathway, is rate limiting (Gore-Langton and Armstrong, 1994). DHEA is converted to androstenedione by the Δ^5 - 3β-hydroxysteroid dehydrogenase: $\Delta^{5\text{-}4}$ -isomerase enzyme complex either in the theca cells (Lacroix *et al.*, 1974; Mc Natty *et al.*, 1984b; Turzillo and Fortune, 1992; Gore-Langton and Armstrong, 1994) or, after crossing the basement membrane, in the granulosa cells (Rubin *et al.*, 1963; Zelezink *et al.*, 1974; Madej, 1980; Jones *et al.*, 1983; Gore-Langton and Armstrong, 1994).

Androstenedione can be converted to testosterone in theca or granulosa cells by

17β-hydroxysteroid dehydrogenase (Figures 2 and 3; Lacroix *et al.*, 1974; Mc Natty *et al.*, 1984b; Payne *et al.*, 1992; Turzillo and Fortune, 1992). Due to a deficiency in thecal 17β-hydroxysteroid dehydrogenase greater amounts of androstenedione are produced compared with testosterone by isolated bovine theca interna tissues or cells (Lacroix *et al.*, 1974; Mc Natty *et al.*, 1984b, Fortune and Hansel, 1985). In granulosa cells, androstenedione is converted to estrone or estradiol by the aromatase P450 enzyme system (Figures 2 and 3; Lacroix *et al.*, 1974; Hansel and Convey, 1983; Richards *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). Hence, androstenedione is an aromatizable androgen. Note, 19-hydroxy-androstenedione (19-OH-A), which will be added to cultures of granulosa cells in this thesis, is the first intermediate formed during conversion of androstenedione to estradiol (Figure 3).

Testosterone is another aromatizable androgen which can be converted into estradiol by the aromatase P450 enzyme system in granulosa cells (Figures 2 and 3; Lacroix *et al.*, 1974; Hansel and Convey, 1983; Richards *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). Note, when testosterone is converted into estradiol, 19-hydroxy-testosterone (19-OH-T), which will be added to cultures of granulosa cells in this thesis, is the first intermediate formed. Alternatively, testosterone can be converted to dihydrotestosterone (DHT), a non-aromatizable androgen (Armstrong and Papkoff, 1976; Hillier and Ross, 1979), by 5 α-reductase in either theca or granulosa cells (Figures 2 and 3; Payne *et al.*, 1992; Gore-Langton and Armstrong, 1994).

In summary, if the aromatase enzyme system is present, granulosa cells can convert DHEA, 19-OH-A, 19-OH-T, but not DHT, into estradiol. Thecal androgens, therefore, are important determinants of granulosa cell estradiol production because they are obligatory precursors for estradiol synthesis (Lacroix *et al.*, 1974; Armstrong and Papkoff, 1976; Leung *et al.*, 1979; Erickson and Magoffin, 1983; Mc Natty *et al.*, 1984b; Payne *et al.*, 1992; Turzillo and Fortune, 1992; Gore-Langton and Armstrong, 1994).

Proliferation

Whether androgens directly regulate granulosa cell proliferation in cows or other species is unknown. However, estradiol stimulates granulosa cell proliferation in rats (Williams, 1940; Goldenberg *et al.*, 1972; Hillier, 1981; Tonetta and Ireland, 1984). This finding implies that androgens indirectly regulate granulosa cell proliferation since they are substrates and thus potential regulators of estradiol production.

Viability

Apoptosis, or programmed cell death is the underlying mechanism of cell loss during ovarian follicular atresia (Hughes and Gorospe, 1991; Tilly *et al.*, 1991; Tilly and Hseuh, 1993; Guthrie *et al.*, 1995a). Granulosa cells, but not theca cells, undergo apoptosis (Peluso *et al.*, 1996). Although information concerning androgens and granulosa cell apoptosis is lacking in cattle, androgens increase granulosa cell apoptosis in rats (Billing *et al.*, 1993). In contrast, estrogens prevent granulosa cell apoptosis (Payne and Hellbaum, 1955; Hillier, 1981; Billing *et al.*, 1993). In addition, when estrogen implants are withdrawn from hypophysectomized female rats, there is increased granulosa cell apoptosis (Billing *et al.*, 1993). Taken together, these findings imply that androgens regulate granulosa cell apoptosis both directly and indirectly through regulation of estrogen production.

Follicular atresia, the enigmatic process whereby oocytes are lost from the ovary by means other than ovulation (Hurwitz and Adashi, 1993), is associated with the death (apoptosis) of granulosa cells (Luciano *et al.*, 1994; Peluso *et al.*, 1996). Little is known about the role of androgens on follicular atresia in cattle. However, androgens cause atresia of ovarian follicles in rats (Payne and Runser, 1958; Louvet *et al.*, 1975; Hillier and Ross, 1979; Hillier *et al.*, 1979; Farookhi, 1981; Billing *et al.*, 1993), guinea pigs (Terranova and Greenwald, 1981), sheep (Scaramuzzi *et al.*, 1977) and humans (Hillier, 1981). In cattle, early follicular atresia is characterized by diminished granulosa cell aromatase enzyme activity (Mc Natty *et al.*, 1984a). In swine, atretic follicles have a reduced capacity to produce estradiol (Westhof *et al.*, 1991; Maxson *et al.*, 1985) despite the finding that enzyme activities for androgen production are maintained (Maxson *et al.*, 1985). Taken together, these findings support the belief of Bagnell *et al.* (1982) that androgen-induced atresia may involve interference in the production and/or action of estradiol, which is anti-atretogenic in ovaries (Payne and Hellbaum, 1955; Hillier, 1981; Billing *et al.*, 1993; Luciano *et al.*, 1994).

In summary, although considerable circumstantial evidence exists implicating androgens in the process of granulosa cell apoptosis (Billing *et al.*, 1993) and follicular atresia (Payne and Runser, 1958; Louvet *et al.*, 1975; Scaramuzzi *et al.*, 1977; Hillier and Ross, 1979; Hillier *et al.*, 1979; Farookhi, 1981; Hillier, 1981; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Evans *et al.*, 1997), the specific role of androgens in this process is poorly understood (Gore-Langton and Armstrong, 1994).

Estradiol production

Aromatase P450 enzyme system: The aromatase P450 enzyme system is comprised of aromatase cytochrome P450 and NADPH-cytochrome P450 reductase (Thompson and Siiteri, 1974; Steinkampf *et al.*, 1987; Gore-Langton and Armstrong, 1994). This enzyme system catalyzes the multi-step reactions involved in conversion of aromatizable androgens, such as androstenedione and testosterone, into the estrogens, estrone and estradiol (Figures 2 and 3; Lacroix *et al.*, 1974; Hansel and Convey, 1983; Richards *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). Acquisition of the aromatase P450 enzyme system in bovine granulosa cells occurs after theca cells differentiate into androgen producing cells (Ireland, 1987; Mc Natty *et al.*, 1984a). Because the aromatase P450 enzyme system is needed for granulosa cells to produce estradiol, examining whether androgens regulate the aromatase enzyme system may be key to understand the process of dominant follicle growth and atresia. Two major ways in which androgens could regulate the aromatase enzyme system are via alterations in gene expression and enzyme activity.

Aromatase P450 mRNA: Aromatase P450 mRNA ($P450_{arom}$ mRNA) is located in granulosa and cumulus cells of bovine follicles (Voss and Fortune, 1993; Xu *et al.*, 1995a). In healthy bovine ovarian follicles, the level of expression increases with follicle size, whereas atretic follicles do not express $P450_{arom}$ mRNA (Xu *et al.*, 1995a). Whether androgens regulate $P450_{arom}$ mRNA in any species is unknown. However, androgens may indirectly affect $P450_{arom}$ mRNA by their involvement in follicular atresia.

Aromatase enzyme activity as measured by estradiol production: Although it is unknown if androgens directly regulate aromatase enzyme activity in bovine granulosa cells, there is evidence of androgen regulation of the aromatase enzyme activity in granulosa cells of rats and humans. Androgens enhance aromatase enzyme activity in rats both *in vivo* (Leung *et al.*, 1978; Goff *et al.*, 1979) and *in vitro* (Lucky *et al.*, 1977; Daniel and Armstrong, 1980; Hillier and De Zwart, 1981; Welsh *et al.*, 1982). However, non-aromatizable 5α-reduced androgens, such as DHT, are competitive inhibitors of granulosa cell aromatase enzyme activity in rats (Hillier and Ross, 1979; Hillier *et al.*, 1979; 1980a) and humans (Schwarzel *et al.*, 1973; Hillier *et al.*, 1980b). These findings support the theory of Hillier *et al.* (1980a) that alterations in the activity of 5α-reductase in combination with changes in thecal androgen production could modulate follicular estradiol synthesis. Furthermore, estradiol production may be influenced by amount of substrate available and variation in the ratio of aromatizable to non-aromatizable androgens as a consequence of changes in 5α-reductase activity (Gore-Langton and Armstrong, 1994).

Androgens may regulate aromatase enzyme activity since they are substrates for estrogens, and estrogens enhance aromatase enzyme activity (Daniel and Armstrong, 1983). Additionally, aromatase enzyme activity is positively correlated with level of estrogens and negatively correlated with ratio of aromatizable to non-aromatizable androgens in human follicular fluid (Yen and Jaffe, 1986).

In summary, although androgens regulate aromatase enzyme activity in granulosa cells of rats and humans, it is unknown if androgens regulate estradiol production in cows. Androgens, in general, increase aromatase enzyme activity, but 5α-reduced androgens are inhibitory. Therefore, the intrafollicular androgen environment, specifically the amounts of each androgen type and ratio of aromatizable to non-aromatizable androgens, may have an important role in regulation of estradiol production.

Thus, based on this review of literature, the following hypothesis will be tested: different types and amounts of androgens alter proliferation, viability, estradiol production, and aromatase enzyme activity *in vitro* of bovine granulosa cells isolated from the first-wave dominant follicle. To test this hypothesis, a serum-free culture system using granulosa cells isolated from the first-wave dominant follicle was developed (Chapter 2) and the following experiments (Chapter 3) conducted:

I) Effects of different types and amounts of androgens on proliferation, viability, and estradiol production;

II) Effects of different types and amounts of androgen on subsequent capacity of granulosa cells to produce estradiol; and

III) Effects of DHT on the production of estradiol by granulosa cells.

Part II

CHAPTER 2

Development and Validation of Techniques

Introduction

The overall objective of this project was to develop a serum-free culture system that could be used reliably to test the hypothesis that different types and amounts of androgen alter proliferation, viability, estradiol production, and aromatase activity *in vitro* of bovine granulosa cells isolated from the first-wave dominant follicle. In previous studies from our laboratory (Jiménez-Krassel and Ireland, unpublished) granulosa cells were isolated from dominant and subordinate follicles on Days 2 to 10 of the bovine estrous cycle (Figure 1). Cells were cultured serum-free in Ham's F-12 medium supplemented with 19-hydroxy-androstenedione (10^{-6} M) for 48 h. After culture, concentrations of estradiol and progesterone in media were determined. Granulosa cells from the first-wave dominant follicle on Days 2 to 5 of the estrous cycle produced more estradiol than progesterone, and greater amounts of estradiol compared with cells from all other follicle types. Because of these results, only granulosa cells isolated from the first-wave dominant follicle during Days 2 to 5 of the estrous cycle were used to test this thesis's hypothesis.

To further enhance and validate the serum-free culture system for bovine granulosa cells from the first-wave dominant follicle, the following four studies were completed. **Study I**: Preliminary studies indicated that bovine granulosa cells isolated from follicles at unknown stages of differentiation and cultured in Endothelial-SFM media produced more estradiol than cells in Ham's F-12 medium (Weber and Ireland, unpublished). Thus, the first study was designed to test whether capacity of bovine granulosa cells from the first-wave dominant follicle to produce estradiol was greater in Endothelial-SFM media compared with Ham's F-12. **Study II**: Trypan blue exclusion dye is used routinely to estimate cell viability, but has not been evaluated for bovine granulosa cells. The second study, therefore, was designed to evaluate whether trypan blue exclusion dye could be used to quantify alterations in number of viable bovine granulosa cells during culture. **Study III**: Viability of bovine granulosa cells cultured for 48 h in Ham's F-12 medium is low (Jiménez-Krassel and Ireland, unpublished). Also, the greater production of estradiol observed for granulosa cells cultured in Endothelial-SFM media compared with Ham's F-12 media (Weber and Ireland, unpublished) could be the result of enhanced cell viability. Thus, the third study was designed to examine the effects of Ham's F-12 and Endothelial-SFM media on viability of bovine granulosa cells. **Study IV**: Since alterations in total number of granulosa cells during the cell culture period could influence estradiol production and confound interpretation of results, use of absorbance to quantify alterations in total number of cells *in vitro* was examined.

Materials and Methods

Reagents and hormones

The following items were obtained from Life Technologies, Inc. (Grand Island, NY): Ham's F-12 liquid medium with L-glutamine, Endothelial-SFM growth medium with L-glutamine (SFM-growth), Endothelial-SFM plating medium with bovine vitronectin and L-glutamine (SFM-plating), penicillin G sodium, streptomycin sulfate, and trypan blue exclusion dye. 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), ethylenediamine tetraacetic acid (EDTA), and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, monobasic phosphate, and dibasic phosphate were obtained from J. T. Baker Chemical Company (Phillipsburg, NJ).

Tissue collection and bovine granulosa cell preparation

Ovaries from beef and dairy cows during Days 2-5 of the estrous cycle were collected from a slaughterhouse (MURCO, Plainwell, MI). Stage of the estrous cycle was determined by morphology of the corpus luteum (Ireland *et al.*, 1980). Pairs of ovaries from each cow were placed in individual bottles containing ice-cold phosphate buffered saline solution (0.01 M monobasic phosphate, 0.04 M dibasic phosphate, 0.1 M sodium chloride, 0.02 M EDTA; pH 7.4) and transported on ice to the laboratory within 4 h of slaughter. The largest follicle per pair of ovaries, which is the first-wave dominant follicle (Fortune, 1993), was dissected, ovarian stroma removed, and the follicle's diameter was recorded. Follicular fluid was aspirated from each follicle by puncturing it with a 22-gauge needle attached to a 3 ml syringe. The follicle shell was then bisected under sterile conditions and placed on a watch glass containing cell isolation medium. Cell isolation medium was either Ham's F-12 or Endothelial-SFM growth (SFM-growth) medium supplemented with penicillin/streptomycin solution (1.5 mg/ml penicillin G sodium and 2.5 mg/ml streptomycin sulfate) as indicated for each study. Granulosa cells were scraped gently from the inner wall of each follicle into cell isolation medium using a bent microspatula. Granulosa cells were pooled, then washed and centrifuged (400 x g for 5 min at 4°C) three times with ice-cold cell isolation medium. Number of cells was estimated using a hemocytometer.

Bovine granulosa cell culture

Ham's F-12 or Endothelial-SFM plating (SFM-plating) medium was used as indicated below for cell culture. Except where noted, 10^5 bovine granulosa cells in 20 μl of cell isolation medium were plated in 96-well Falcon Primaria culture plates (Becton Dickinson and Co., Lincoln Park, NJ) containing 180 μl of medium previously equilibrated at 37°C and supplemented with penicillin/streptomycin solution (1.5 mg/ml penicillin G sodium and 2.5 mg/ml streptomycin sulfate) and 19-OH-A (1×10^{-6} M). Granulosa cells were incubated at 37°C in a humidified atmosphere (5 % CO_2 and 95 % air) for 18 or 24 h. After incubation, cells were detached from culture wells by gently flushing them with the existing media in the wells. Media and cells were collected from each well, placed in individual 1.5 ml Eppendorf tubes, and centrifuged (400 x g for 10 min at 4°C) to form pellets of cells. Media were then carefully removed without disturbing the pellet and stored at -20°C until assayed for estradiol. Fresh, ice-cold cell isolation medium (200 μl) supplemented with penicillin/streptomycin solution was added to the cell pellet and cells were resuspended then stored on ice until viability was assessed.

Viability assessment

Viability of granulosa cells after isolation from follicles (initial) and viability after culture (final) were determined by mixing 200 μl of a 1:10 dilution of the initial cell suspension, or 200 μl of a cell suspension that had previously been stored on ice after completion of the culture period, with 200 μl of a 1:5 dilution of 0.4% trypan blue exclusion dye. The mixture was incubated at room temperature for 1 minute. An aliquot was then placed on a hemocytometer and examined under the microscope. Stained cells were considered dead. Viability was expressed as percent of viable (non-stained) cells per total (stained + non-stained) cells in a well.

Estradiol determination

Concentrations of estradiol were measured in media by radioimmunoassay using previously validated (Turzillo and Fortune, 1990; Ireland *et al.*, 1994), commercial kits (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity of the assay was 0.5 pg/ml. Cross-reactivity of estradiol antiserum with estrone is 12.5% and less than 1% with other steroids.

Study I: Effects of different culture media supplemented with 19-OH-A on capacity of bovine granulosa cells from first-wave dominant follicles to produce estradiol

To determine if culture medium and number of cells plated affected capacity of bovine granulosa cells to produce estradiol, different numbers of granulosa cells per culture well were incubated in two different media. Specifically, granulosa cells were collected and processed for culture as described above, except half of the follicle shell was placed on a watch glass containing either Ham's F-12 medium or SFM-growth medium, and granulosa cells were isolated, washed, and counted in each medium separately. Cells were plated at different concentrations (0.25×10^5, 0.50×10^5, 1×10^5, 2.5×10^5, 5×10^5, and 10×10^5 cells / well) in culture wells containing either Ham's F-12 medium or SFM-plating medium. One group of 5×10^5 cells for each medium received no androgen. At the end of the 24 h culture, media were collected and assayed for estradiol.

Study II: Evaluation of trypan blue exclusion dye

To determine if trypan blue exclusion dye can be used to assess alterations in bovine granulosa cell viability, cells (10^5 / tube) were incubated with different concentrations of sodium azide (0, 0.005, 0.50, 2.50, 5.00, 25.0, 50.0, and 100.0 M) in 1.5 ml Eppendorf tubes. [Sodium azide is a well established cell poison (Bortkiewicz *et al.*, 1988; Telford *et al.*, 1991; Vaux and Häcker, 1995) that disrupts the electron-transport chain in mitochondria (Dawson *et al.*, 1993).] Cells were incubated for 1 h (37°C, 5 % CO_2, 95 % air) in sodium azide then pelleted by centrifugation (400 x g for 5 min at 4°C). The supernatant was removed, 200 μl of ice-cold SFM-growth medium was added, and cells were resuspended and stored on ice until final viability was determined.

Study III: Effects of culture media on viability of bovine granulosa cells

To determine if culture medium and number of cells plated affected viability of bovine granulosa cells, initial viability of granulosa cells after isolation from follicles was compared with viability after the 24 h culture period. Granulosa cells were collected, processed, and different numbers of cells (0.25×10^5, 0.50×10^5, 1×10^5, 2.5×10^5, 5×10^5, and 10×10^5 cells / well) cultured in two different media (Ham's F-12 and SFM), as described in Study I.

Study IV: Use of absorbance to quantify alterations in granulosa cell number during culture

The rationale for this study is based on results by Mohler *et al.* (1996) and preliminary data (not shown) that absorbance of light at a wavelength of 405 nm (A_{405}) can be used to quantify alterations in total numbers of cells. Use of absorbance to determine alterations in cell number, therefore, would be a simple, non-invasive assay which could be performed while cells remained in culture. In addition, alterations in cell numbers could be monitored several times throughout the culture period without cell loss due to transfer of cells from culture wells to other containers for assays. Finally, the need for separate culture wells to determine cell number could be eliminated.

To validate the absorbance method (Mohler *et al.,* 1996), the following experiments were completed: a) relationship of number of bovine granulosa cells to absorbance at 405 nm; and b) to determine if temperature, cell size, or androgens altered absorbance measurements at 405 nm. Affect of temperature was examined because the decrease in temperature occurring when a culture plate is removed from the incubator until absorbance readings are made on the microplate reader may change the pH (Weast, 1989) of medium containing phenol red, thus altering the medium's color, and in turn, its absorbance properties. Since cells in culture may also change size as an effect of experimental treatment or apoptosis (Ellis *et al.*, 1991; Wyllie, 1993), affect of cell size on absorbance at 405 nm was investigated. Finally, although peak absorbance values for steroid hormones are between 225 nm and 287 nm (Weast, 1989), indicating they should not affect absorbance measurements at 405 nm, the effect of different concentrations and types of androgens were investigated to ensure that alterations in absorbance readings at

405 nm reflected changes in cell number.

A. Relationship of number of bovine granulosa cells to absorbance at 405 nm

To determine if changes in cell number and absorbance at 405 nm were correlated, different numbers (0.5×10^5, 1×10^5, 2×10^5, and 2.5×10^5) of granulosa cells were plated per culture well and incubated for 24 h. Absorbance at 405 nm was then determined using a microplate reader (Bio-Rad model #3550; wavelength filter set for 405 nm). Absorbance of granulosa cells was calculated by subtracting the background absorbance values for wells containing only media (20 μl SFM-growth medium + 180 μl SFM-plating medium / well) from the absorbance values of wells containing media and granulosa cells.

B. Effects of temperature, cell size, and androgens on absorbance at 405 nm

To evaluate the effect temperature has on absorbance, a mixture of SFM-growth (20 μl) and SFM-plating (180 μl) media without cells was placed in a random fashion in 13 wells of two 96-well Primaria culture plates. One plate was kept at 37°C for 18 h, whereas the other was kept at room temperature for 18 h. After the 18 h incubation period ended, absorbance readings (A_{405}) were at 5 min intervals for 60 min, 15 min intervals for the next 30 min, and at 30 min intervals for the next 90 min.

To determine if cell size affected absorbance, granulosa cells were isolated and cultured in SFM media without androgen and supplemented with different concentrations (0, 0.3, 0.5, 1.0, or 2.0 M) of sodium chloride. Sodium chloride was used to create hypertonic conditions which should cause granulosa cells to decrease in size. Absorbance readings (A_{405}) were taken 1 h after addition of sodium chloride. Background values for wells containing only media (20 μl SFM-growth + 180 μl SFM-plating media / well) with different concentrations of sodium chloride were subtracted from corresponding wells containing media, sodium chloride and cells. Cells were then examined under a light microscope. The number of relatively small cells per 20 cells in a random area was recorded for each well.

To determine if androgens altered absorbance, SFM-plating medium (without cells) supplemented with different types (19-OH-A, 19-OH-T, DHEA, DHT, or no androgen) and amounts (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) of androgens were placed in culture wells (200 μl medium / well) and incubated for 18 h. Absorbance measurements (A_{405}) were taken at 1 h and 18 h to determine if absorbance was altered over time.

Statistical analysis

All analyses were performed using SPSS Base 8.0 (SPSS Inc., Chicago, IL). Experimental data are expressed as arithmetic means (± SEM) of measurements of triplicate culture wells or aliquots. With the exception of Study IV, studies were replicated at least twice with different pools of bovine granulosa cells isolated from ovaries obtained during separate trips to an abattoir. Two-way analysis of variance (ANOVA) was used to test the effects of: culture media and number of granulosa cells on estradiol production (Study I) and cell viability (Study III); incubation temperature and time on alterations in absorbance at 405 nm (Study IV); and types and doses of androgens on alterations in absorbance at 405 nm (Study IV). One-way ANOVA was used to test the effects of sodium azide concentration on trypan blue uptake by cells (Study II) and the effects of sodium chloride concentration on absorbance measurements at 405 nm and percent of small cells (Study IV). Means were compared by the Bonferroni t-test. Pearson's correlation test was used to determine the relationship between number of granulosa cells and absorbance at 405 nm (Study IV). Unless stated otherwise, significance was $P < 0.05$.

Results

Study I: Effects of different culture media supplemented with 19-OH-A on capacity of bovine granulosa cells from first-wave dominant follicles to produce estradiol

Granulosa cells cultured in SFM-plating medium produced 40 to 95 fold ($P<0.05$) more estradiol than those in Ham's F-12 medium (Figure 4). In SFM-plating medium estradiol production per cell increased ($P<0.05$) from $0.25\text{x}10^5$ to $1\text{x}10^5$ cells per well, remained constant from $1\text{x}10^5$ to $5\text{x}10^5$ cells per well, and then decreased ($P<0.05$) from $5\text{x}10^5$ to $10\text{x}10^5$ cells per well (Figure 4). In contrast, maximum estradiol production for cells cultured in Ham's F-12 medium was at $10\text{x}10^5$ cells per well. Estradiol production for cells cultured in either medium without androgen (NA) was negligible.

Study II: Evaluation of trypan blue exclusion dye

Viability of bovine granulosa cells was the same initially (t=0) and after 1 h incubation (t=1) for cells in medium without sodium azide (Figure 5). However, after 1 h in culture, viability of granulosa cells decreased ($P<0.05$) as amount of sodium azide increased.

Study III: Effects of culture media on viability of bovine granulosa cells

Granulosa cell viability was initially higher ($P<0.05$) for cells isolated in SFM-growth media compared with Ham's F-12 medium (Figure 6). Cell viability was stable during the 24 h culture period for cells in SFM-plating medium supplemented with 19-OH-A. In contrast, viability of cells in Ham's F-12 medium decreased ($P<0.05$) compared with initial viability. Viability of cells cultured in either medium without 19-OH-A (NA) was lower ($P<0.05$) compared with cells cultured in media supplemented with 19-OH-A. Number of granulosa cells plated per well had no consistent effect on cell viability.

Study IV: Use of absorbance to quantify alterations in number of granulosa cells during culture

A. Relationship of number of bovine granulosa cells to absorbance at 405 nm

Absorbance at 405 nm was correlated positively ($P<0.05$) with increasing number of granulosa cells (Figure 7).

B. Effects of temperature, cell size, and androgens on absorbance at 405 nm

As culture medium cooled from 37°C to room temperature, absorbance decreased ($P<0.05$) (Figure 8). In contrast, absorbance of medium at room temperature (Figure 8) was stable ($P>0.10$) and lower ($P<0.05$) compared with medium at 37°C.

As concentration of sodium chloride increased, the percentage of relatively small bovine granulosa cells increased ($P<0.05$) concurrently with a decrease ($P<0.05$) in absorbance at 405 nm (Figure 9).

Initial absorbance was higher ($P<0.05$) for 19-OH-T and DHEA (except 10^{-6} M) compared with no androgen, 19-OH-A (except 10^{-8} M), and DHT (Figure 10). Final absorbance was higher ($P<0.05$) for 19-OH-T, DHEA, and 10^{-8} M and 10^{-7} M doses of DHT compared with no androgen, 19-OH-A, and 10^{-6} M and 10^{-5} M doses of DHT. Final absorbance for 19-OH-T, DHEA, and DHT was higher ($P<0.05$) than initial absorbance (Figure 10). There was no consistent dose effect of androgens on absorbance.

Figure 4. Effect of number of bovine granulosa cells on estradiol production per cell. Pairs of ovaries from cows during Days 2-5 of the estrous cycle were collected from a slaughterhouse. The largest follicle per pair of ovaries, which is the first-wave dominant follicle (Fortune, 1993), was isolated. Follicular fluid was aspirated from the follicle, the follicle was cut in half, and each half was placed on a watch glass containing either Ham's F-12 medium or Endothelial-SFM growth medium. Granulosa cells were gently scraped into each medium. Cells were plated at different concentrations (x-axis; number x 10^5) in a 96-well culture plate containing either Ham's F-12 medium (■) or Endothelial-SFM plating medium (SFM; □) supplemented with 10^{-6} M 19-hydroxy-androstenedione, except for two groups of $5x10^5$ cells which received no androgen (NA). Cells were incubated for 24 h. Media and cells were then collected and separated as explained in Methods. Radioimmunoassay was used to determine concentration of estradiol in media. To determine estradiol production per cell (y-axis), the total amount of estradiol per well was divided by cell number. Each bar depicts mean (± SEM) values for 6 replicates from a single experiment.

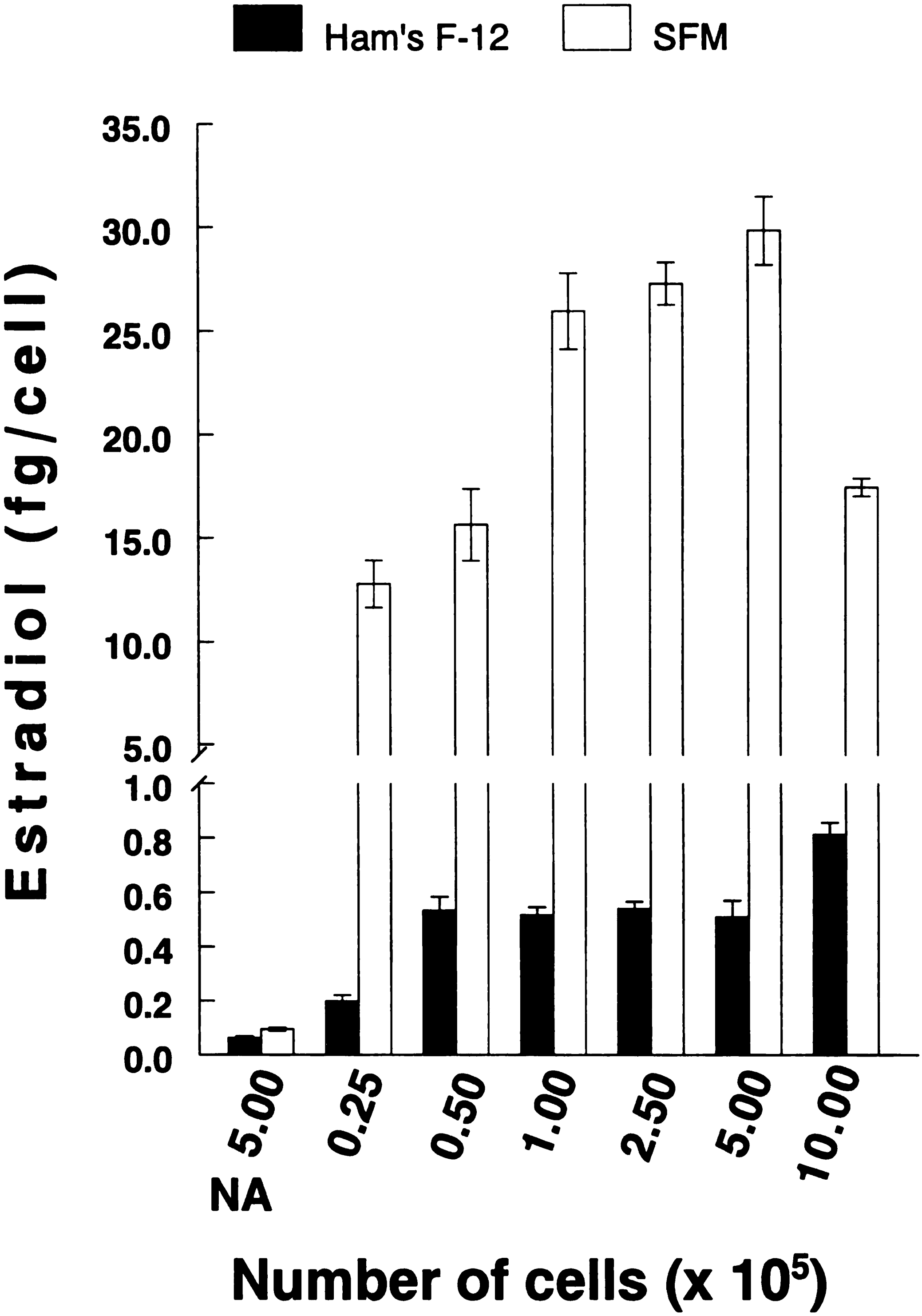

Ham's F-12
SFM
Estradiol (fg/cell)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
1.0
0.8
0.6
0.4
0.2
0.0
5.00
0.25
0.50
1.00
2.50
5.00
10.00
NA
Number of cells (x 10⁵)

Figure 5. Effect of sodium azide on viability of bovine granulosa cells.

Granulosa cells were isolated and prepared for culture as explained in Figure 4's legend. Cells were pipetted into 1.5 ml microcentrifuge tubes (10^5 cells / tube) containing Endothelial-SFM growth medium supplemented with different concentrations of sodium azide (NaN_3) (x-axis). Initial (t = 0) and final (t = 1) viability (y-axis) were assessed using trypan blue exclusion dye. Bars represent mean (± SEM) values for 3 replicates from a single experiment.

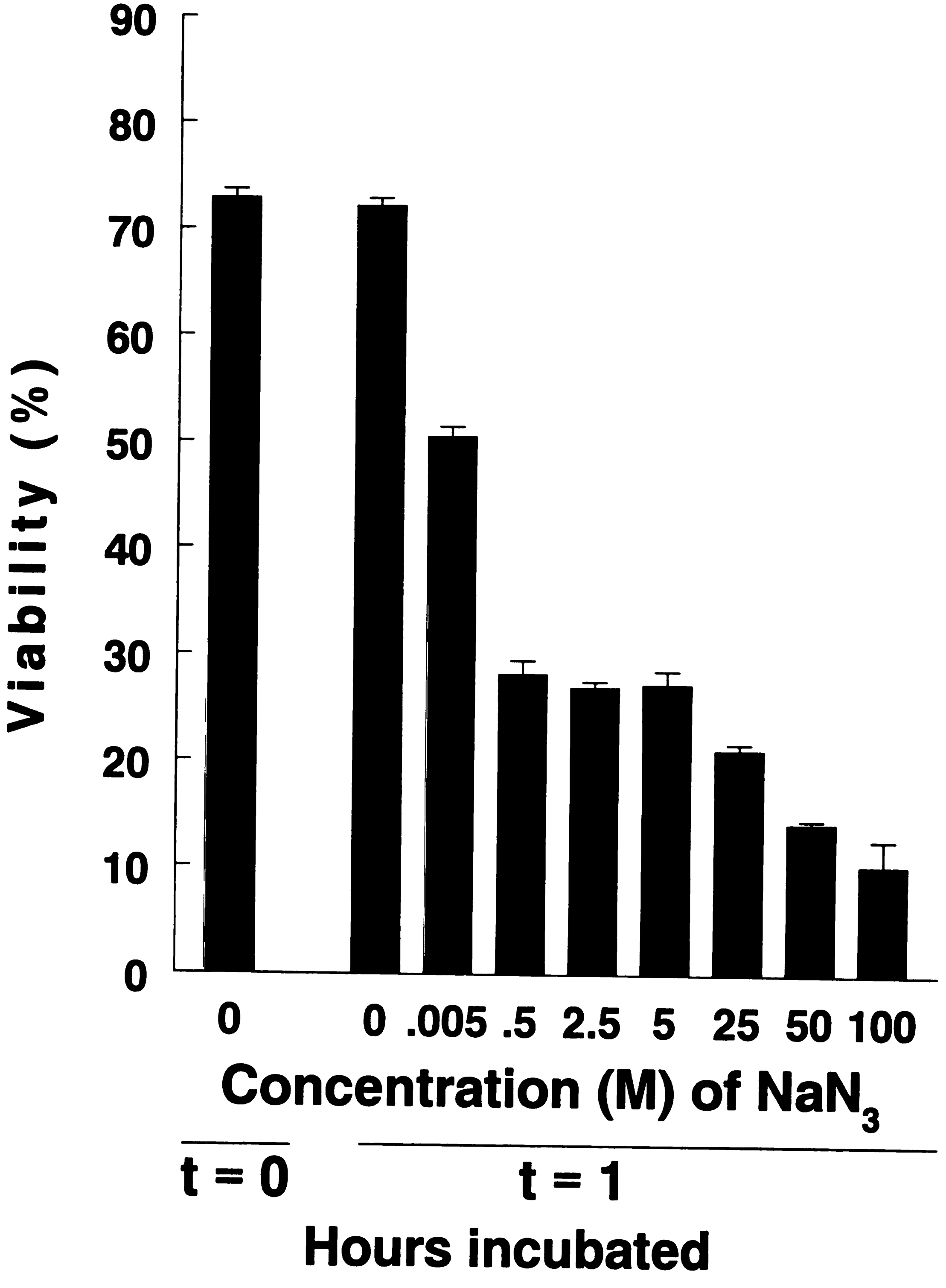

90
80
70
60
50
40
30
20
10
0
Viability (%)
0
0
.005
.5
2.5
5
25
50
100
Concentration (M) of NaN3
t = 0
t = 1
Hours incubated

Figure 6. Effects of culture media and number of bovine granulosa cells on cell viability. Granulosa cells were isolated and prepared for culture as explained in Figure 4's legend. Cells were plated at different concentrations (x-axis; number x 10^5) in a 96-well culture plate containing either Ham's F-12 medium (■) or Endothelial-SFM plating medium (SFM; □) supplemented with 10^{-6} M 19-hydroxy-androstenedione, except for two groups of 5×10^5 cells which received no androgen (NA). Cells were incubated for 24 h. Media and cells were then collected and separated as explained in Methods. Initial and final viability (y-axis) were assessed using trypan blue exclusion dye. Each bar depicts mean (± SEM) values for 3 replicates from a single experiment.

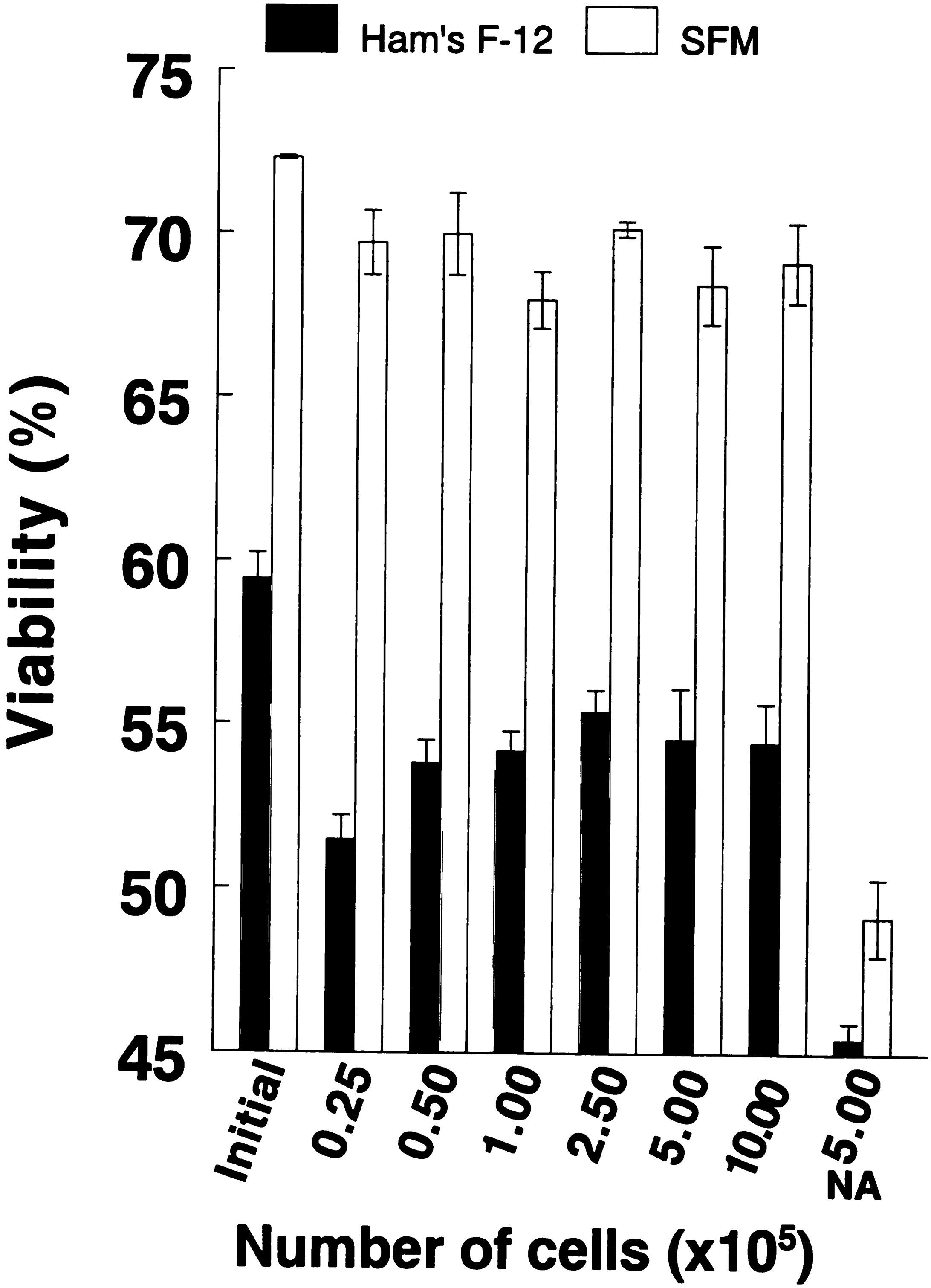

Ham's F-12
SFM
75
70
65
60
55
50
45
Viability (%)
Initial
0.25
0.50
1.00
2.50
5.00
10.00
5.00
NA
Number of cells (x10⁵)

Figure 7. Relationship of number of bovine granulosa cells to absorbance at 405 nm. Granulosa cells were isolated and processed for culture as explained in Figure 4's legend. Cells were plated at different concentrations (x-axis) in a 96-well culture plate containing Endothelial-SFM plating medium supplemented with 10^{-6} M 19-hydroxy-androstenedione. Absorbance readings at 405 nm (A_{405}) were taken using a microplate reader after completion of the 24 h incubation. A_{405} was selected based on a study by Mohler *et al.*, (1996) demonstrating that absorbance increases with increased cell number and previous results showing the absorbance values for granulosa cells were highest at A_{405}. Absorbance of granulosa cells was determined by subtracting background absorbance for wells containing only media. Each symbol represents mean (± SEM) values for 3 replicates for a single experiment. In follow up studies, results were similar for 3 additional experiments.

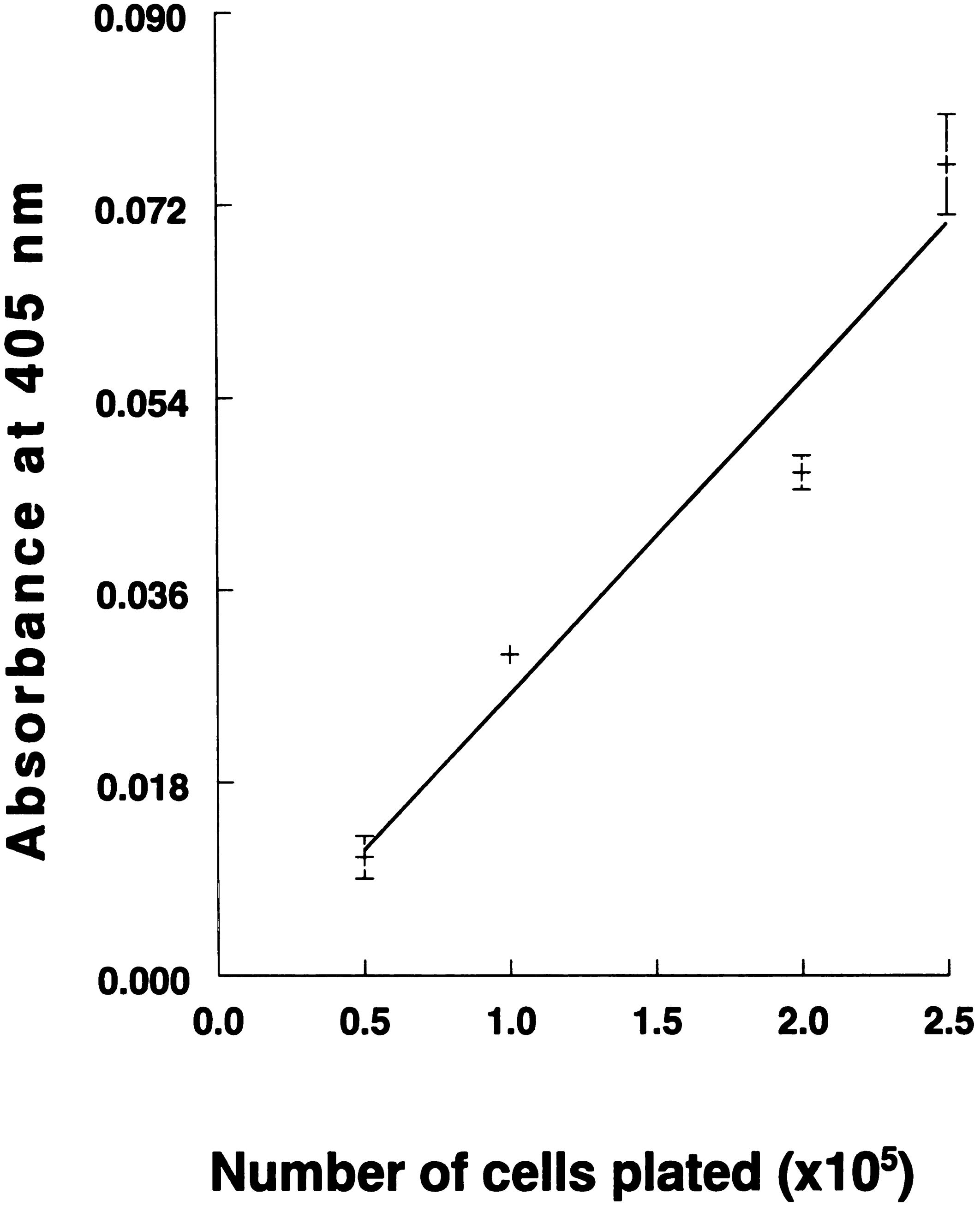

0.090
0.072
0.054
0.036
0.018
0.000
0.0
0.5
1.0
1.5
2.0
2.5
Absorbance at 405 nm
Number of cells plated (x10⁵)

Figure 8. Effect of temperature on absorbance at 405 nm.

Endothelial-SFM medium (20 μl growth medium + 180 μl plating medium / well) without cells was placed in a random fashion in wells of two 96-well Primaria culture plates. Plate 1 (37° to RT; +) was incubated at 37°C for 18 h and Plate 2 (RT; ●) was left at room temperature (RT, approximately 20°C) for 18 h. After incubation, absorbance readings at 405 nm (A_{405}) were then taken at various intervals (x-axis) using a microplate reader. After the initial reading (t=0 h), Plate 1 was not returned to the incubator. Each symbol represents mean (± SEM) value for 13 replicates from a single experiment.

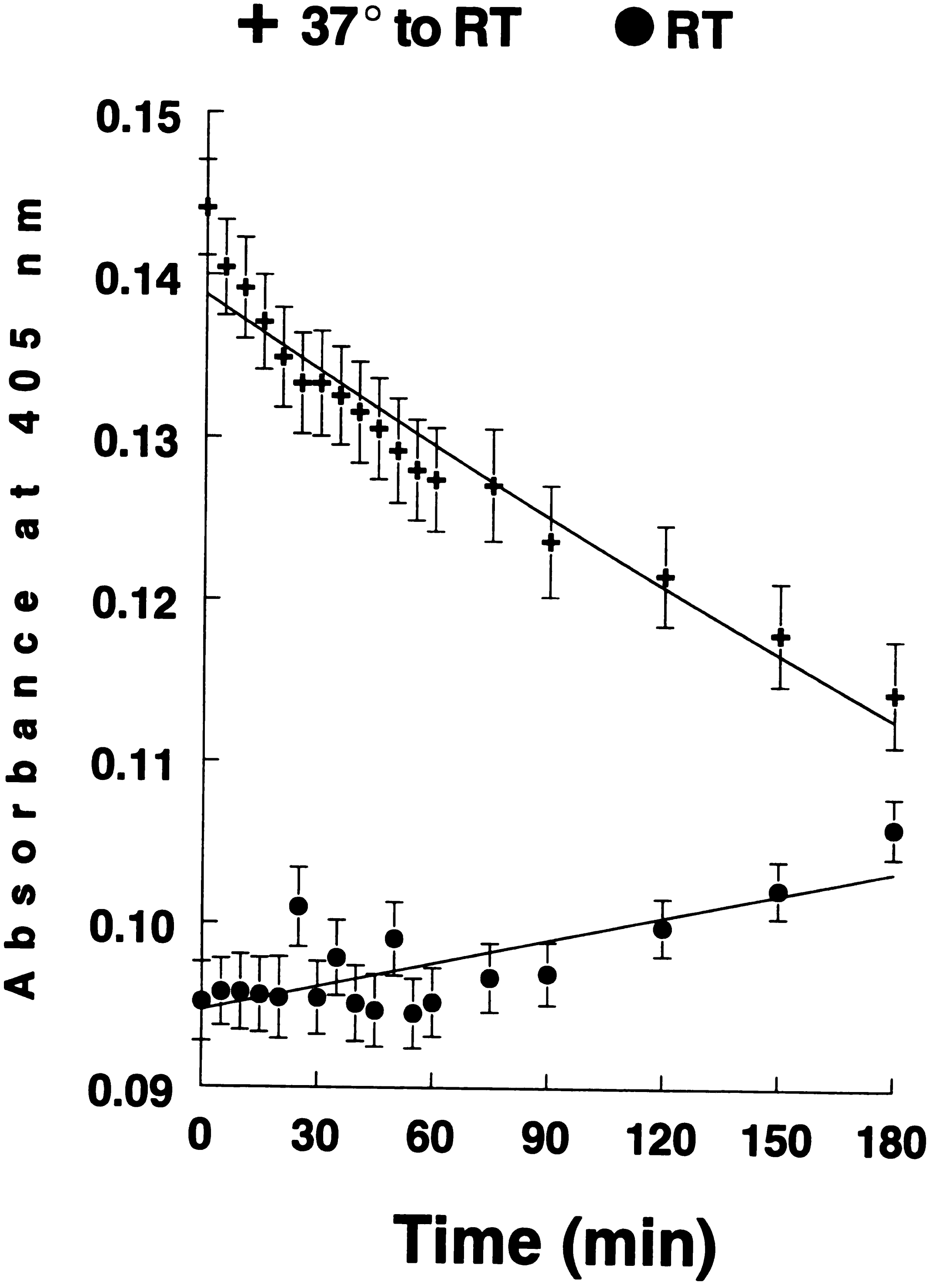

+ 37° to RT
● RT
Absorbance at 405 nm
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0
30
60
90
120
150
180
Time (min)

Figure 9. Effect of sodium chloride on size and absorbance of bovine granulosa cells. Granulosa cells were isolated and processed for culture as explained in Figure 4's legend. Cells were plated in a 96-well culture plate (10^5 cells / well) containing Endothelial-SFM plating medium supplemented with 0 (Control), 0.3, 0.5, 1.0, or 2.0 M sodium chloride (NaCl, x-axis). Cells were incubated for 1 h then an absorbance reading at 405 nm was taken using a microplate reader (left y-axis). Cells were then examined under a light microscope. The percentage of relatively small cells (right y-axis) was determined by dividing number of small cells per 20 cells in a random area in the microscopic field per well by 20 and multiplying that value by 100. Each solid bar represents the mean (± SEM) absorbance values for 4 replicates. Each hatched bar represents the mean (± SEM) values of small cells for 4 replicates from a single experiment.

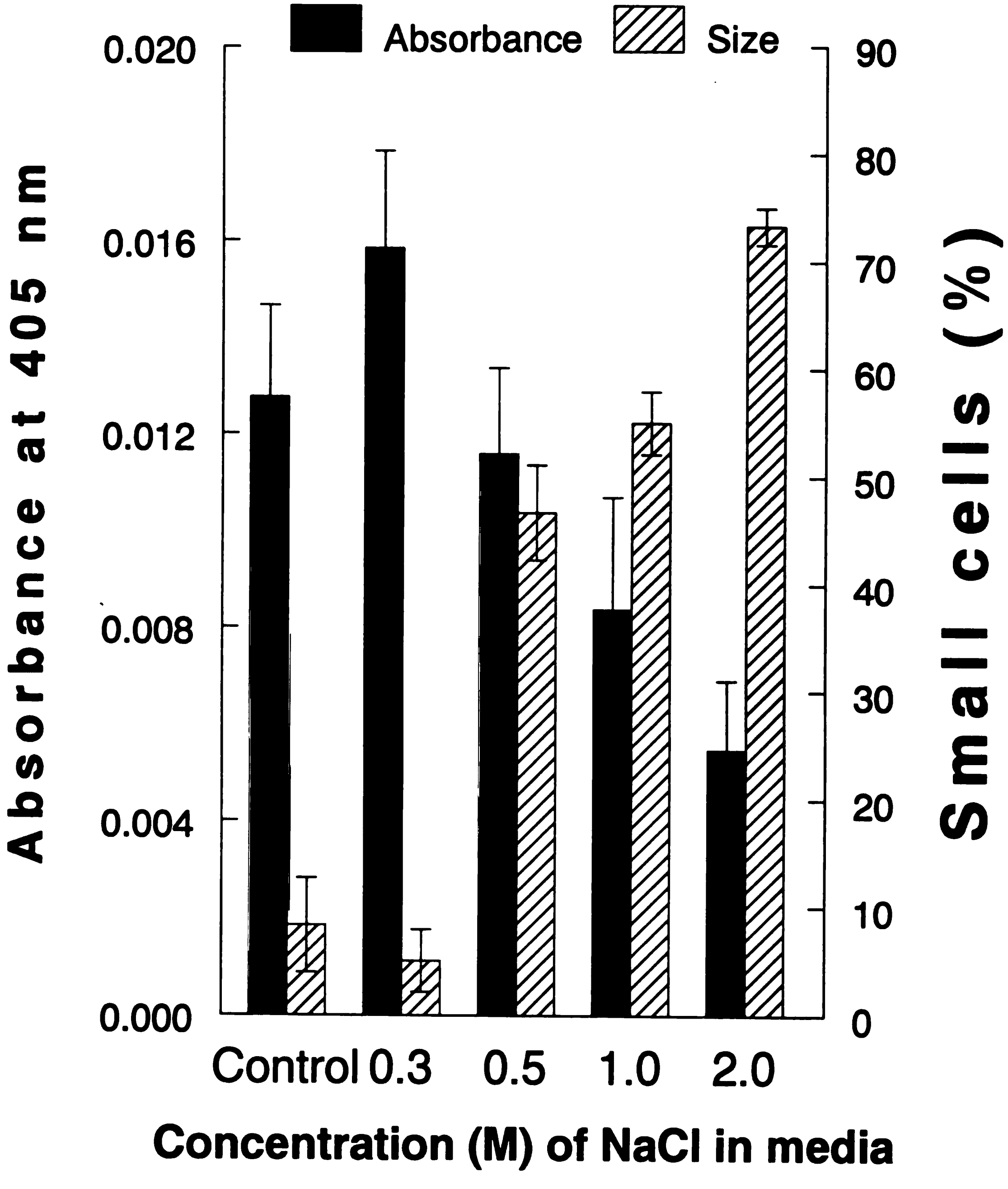
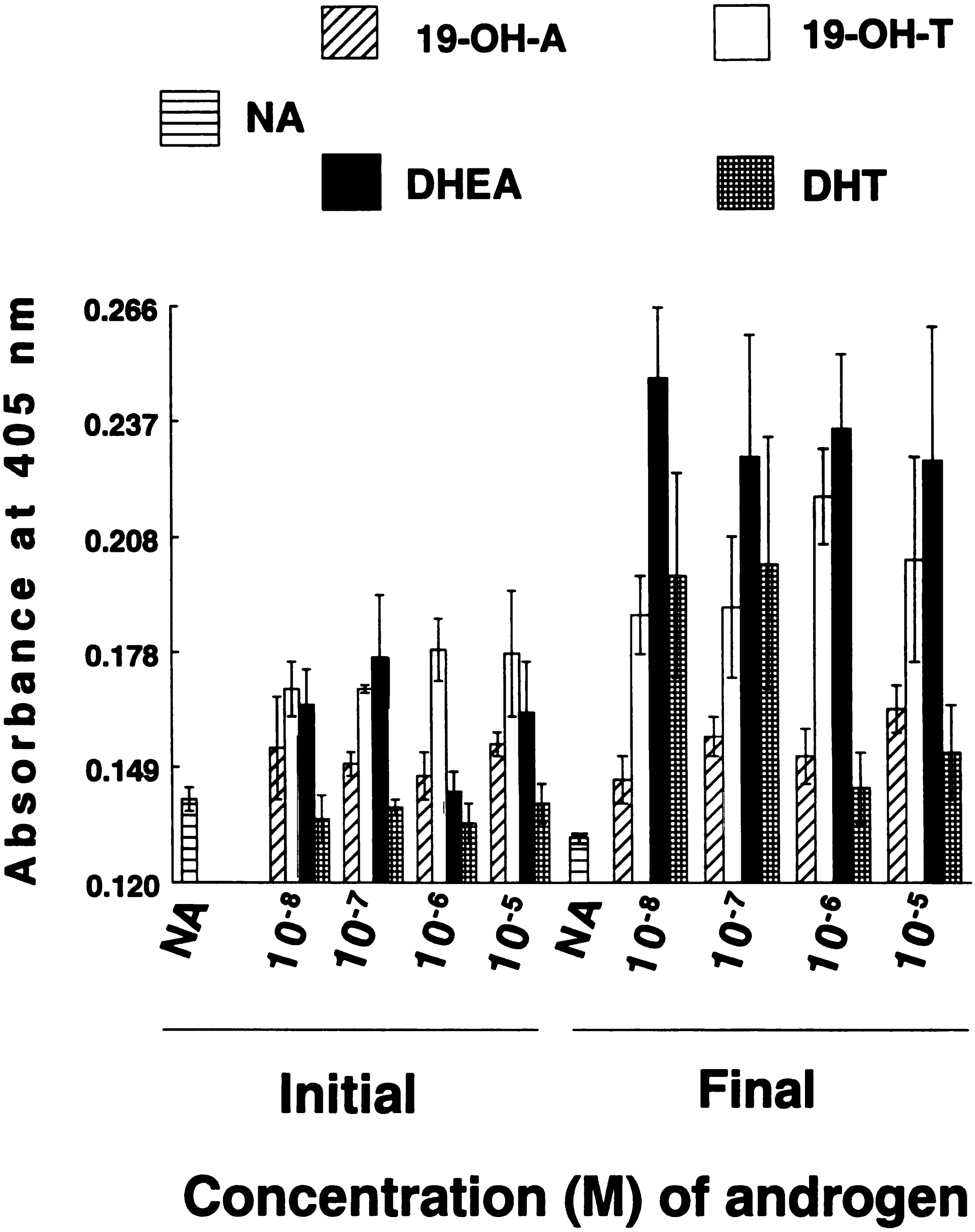

Absorbance
Size
Absorbance at 405 nm
0.020
0.016
0.012
0.008
0.004
0.000
Small cells (%)
90
80
70
60
50
40
30
20
10
0
Control
0.3
0.5
1.0
2.0
Concentration (M) of NaCl in media

Figure 10. Effect of different types and amounts of androgens on absorbance at 405 nm. Endothelial-SFM media (20 μl growth medium + 180 μl plating medium/ well) containing no cells and supplemented with different concentrations (x-axis) of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), or no androgen (NA) were placed in a 96-well culture plate and incubated for 18 h. Absorbance readings at 405 nm (A_{405}) were taken 1 h after start of the incubation (Initial) and after incubation ended (Final). Bars represent mean (± SEM) values for 4 replicates from a single experiment.


19-OH-A
19-OH-T
NA
DHEA
DHT
Absorbance at 405 nm
0.266
0.237
0.208
0.178
0.149
0.120
NA
10-8
10-7
10-6
10-5
NA
10-8
10-7
10-6
10-5
Initial
Final
Concentration (M) of androgen

Discussion

The most significant findings of the present study are that: a) bovine granulosa cells from first-wave dominant follicles cultured in SFM-plating medium produced markedly more estradiol compared with those in Ham's F-12 medium; b) trypan blue exclusion dye can be used reliably to estimate bovine granulosa cell viability; c) bovine granulosa cells cultured in SFM-plating medium were more viable than those in Ham's F-12; and d) absorbance at 405 nm was an unreliable method to measure alterations in cell number.

The reason that estradiol production and viability were higher for granulosa cells cultured in SFM-plating medium compared with those in Ham's F-12 is unknown. Although formulations for SFM media are proprietary, bovine vitronectin, a cell attachment factor (Hoshi *et al.*, 1991; Gorfien *et al.*, 1993), is in SFM-plating medium. Since mammalian cells usually grow best when attached to a surface (Rappaport *et al.*, 1960), this may explain why viability of bovine granulosa cells was stable during the 24 h culture period and greater in SFM-plating medium verses Ham's F-12. In support of a positive effect of vitronectin, vitronectin enhances attachment of bovine granulosa cells to culture wells (Hoshi *et al.*, 1991). In addition, bovine granulosa cells that attach to culture plates produce more estradiol *in vitro* compared with unattached cells (Metcalf, 1982). SFM-plating medium, therefore, may cause granulosa cells to attach better to culture wells compared with Ham's F-12 medium, which is devoid of attachment factors, and thus produce more estradiol compared with cells cultured in Ham's F-12 medium. Because more cells cultured in SFM-plating medium were viable compared with those in Ham's F-12 medium, this could also contribute to the higher estradiol production in

SFM-plating medium. The higher viability of bovine granulosa cells in SFM-plating medium compared with Ham's F-12 may also be due to the presence of growth factors, such as insulin and low density lipoprotein. For example, insulin, which is commonly used as a constituent of commercial serum-free media (Jean Donovan, Life Technologies, Inc., personal communication), is a requisite for growth of many cell lines (Hayashi *et al.*, 1978; Mather and Sato, 1979;) and is important in maintaining normal physiological functions of porcine (May and Schomberg, 1981) and bovine (Savion *et al.*, 1981; Hoshi *et al.*, 1991; Gutiérrez *et al.*, 1997) granulosa cells *in vitro*. In addition, lipoproteins, such as low density lipoprotein (LDL), are common constituents available in commercial serum-free media (Jean Donovan, Life Technologies, Inc., personal communication). LDL maintains porcine granulosa cell survival (Buck and Schomberg, 1987) and increases bovine granulosa cell proliferation (Hoshi *et al.*, 1991) *in vitro*. If insulin and/or LDL or other growth factors are in SFM media, they could also explain the increase in granulosa cell viability and estradiol production for granulosa cells in SFM verses Ham's F-12 media.

Viability was the same for the different number of bovine granulosa cells in SFM-plating medium. Thus, the decreased estradiol production observed when number of cells exceeded $5x10^5$ may be due to limited availability of androgen substrate. In future studies, 10^5 cells per well cultured in SFM-plating medium supplemented with 10^{-6} M 19-OH-A will be used because estradiol production on a per cell basis was similar compared with larger numbers of cells. Also, by using 10^5 cells per well, a single first-wave dominant follicle can be expected to provide enough cells for at least 50 culture wells (Ireland and Roche, 1983a).

A reliable method to quantify cell viability is important because hormonal treatments of cells could affect viability, thus influencing interpretation of experimental results. For example, if treated cells produced less estradiol compared with controls, was it because estradiol production, cell viability, or both were reduced? Trypan blue exclusion dye is commonly used to determine cell viability. This assay is based on the assumption that dead cells have "damaged" membranes which allow charged dye molecules access to the cytoplasm (Cook and Mitchell, 1989). Once dye enters the cell, it is stained blue, and the cell is considered dead. Dye does not enter viable cells with intact membranes, thus viable cells remain clear. To confirm that trypan blue exclusion dye could be used to measure alterations in bovine granulosa cell viability, different concentrations of sodium azide were used to poison (Bortkiewicz *et al.*, 1988; Telford *et al.*, 1991; Vaux and Häcker, 1995) granulosa cells. The results of this study showed that increased amounts of sodium azide decreased viability of bovine granulosa cells causing sodium azide-treated, putatively dead granulosa cells to take up the trypan blue dye. Hence, trypan blue exclusion dye was considered a reliable qualitative and quantitative marker to assess viability of bovine granulosa cells.

It is common to use a spectrophotometer to monitor growth of bacteria (Sambrook *et al.*, 1989), but is not a typical practice with tissue culture cells. Mohler *et al.* (1996) developed a spectrophotometric assay to quantify cell number in tissue culture. This assay required cells to be transferred from culture wells to cuvettes and kept in suspension while absorbance was measured. However, when cells are transferred from culture wells to another container loss of cells is unavoidable due to a portion of cells remaining in the culture wells and sticking to pipette tips. Therefore, a spectrophotometric assay in which cells remained in culture wells would be advantageous. In the present study, absorbance at 405 nm increased in a linear manner with increasing granulosa cell numbers. Morever, when water was used to lyse cells absorbance decreased to zero (data not shown). However, the signal to noise ratio was very high indicating that slight variations in background absorbance could markedly influence absorbance for cells. In addition, temperature, cell size, and androgens influenced absorbance. The reason temperature altered absorbance may be due to a flux in pH (Weast, 1989) and thus change in color of SFM medium since it contains phenol red. As granulosa cells shrunk absorbance decreased probably because there was less cell area to absorb light compared with normal sized cells. It is unknown why androgens altered absorbance. To my knowledge, peak absorbance values for 19-OH-A, 19-OH-T, DHEA, and DHT have not been reported. However, since peak absorbance for most other steroids is between 225 nm and 287 nm (Weast, 1989), it is assumed that the androgens used in the present studies would have similar absorbance peaks. Therefore, androgens should not affect absorbance measurements at 405 nm. Nevertheless, androgens may interact with some unknown component in SFM-plating medium to produce a compound that absorbs light at 405 nm. Because of these aforementioned problems, absorbance at 405 nm was considered unreliable to determine alterations in number of cells during culture. Consequently, cell number in culture will be ascertained in future studies using a commercially available DNA assay kit.

Summary and Conclusions

In summary: a) bovine granulosa cells from the first-wave dominant follicle cultured in SFM-plating medium are more viable and produce markedly more estradiol compared with cells cultured in Ham's F-12 medium; b) 10^5 cells in SFM-plating medium produced the most estradiol per cell; c) trypan blue exclusion dye can be reliably used to estimate alterations in bovine granulosa cell viability; and d) measuring absorbance at 405 nm to quantify cells is unreliable. Consequently, it is concluded that bovine granulosa cells could be cultured in SFM media, viability assessed by trypan blue exclusion dye, and proliferation monitored by DNA analysis to test the hypothesis that different types and amounts of androgen alter growth, viability, estradiol production, and aromatase activity *in vitro* of bovine granulosa cells isolated from the first-wave dominant follicle.

Chapter 3 will describe the effects of different types and amounts of androgens on bovine granulosa cell proliferation, viability, estradiol production, and aromatase enzyme activity using the aforementioned cell culture parameters.

Part III

CHAPTER 3

Introduction

Estradiol is associated positively with selection, growth, and dominance and negatively associated with atresia of ovarian follicles in cattle. Specifically, there is an increase in estradiol production by follicles that escape atresia during each wave of dominant follicle growth (Figure 1). Granulosa cells in the dominant follicle produce more estradiol than those in subordinate or atretic follicles (Figure 1; Padmanabhan *et al.*, 1984; Ireland and Roche, 1987; Fortune, 1994). These observations imply that ovarian estradiol production has an important role in regulation of dominant follicle growth in the cow.

Nevertheless, without theca cell production of androgens, which provides substrate for granulosa cells to synthesize estradiol (Figures 2 and 3), there would be no estradiol produced. The major androgens aromatized to estradiol are androstenedione and testosterone, which are converted into estradiol by the aromatase enzyme system in granulosa cells (Figures 2 and 3; Lacroix *et al.*, 1974; Hansel and Convey, 1983; Richards *et al.*, 1987; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). The ability of bovine granulosa cells to aromatize androgens increases as a function of follicle maturity (Mc Natty *et al.*, 1979b; Hillier, 1981). Increased availability of aromatizable androgens from theca cells may also be responsible for increased estradiol production by granulosa cells (Tian *et al.*, 1995). In contrast, other thecal androgens, such as dihydrotestosterone (DHT), inhibit aromatase activity competitively in granulosa cells from rats (Hillier and Ross, 1979; Hillier *et al.*, 1979; Hillier *et al.*, 1980a) and humans (Schwarzel *et al.*, 1973; Hillier *et al.*, 1980b). Whether DHT inhibits aromatase activity in cattle is unknown.

Follicular atresia is associated with the death of granulosa cells (Luciano *et al.*, 1994; Peluso *et al.*, 1996). There is considerable evidence implicating androgens in the process of granulosa cell apoptosis (Billing *et al.*, 1993) and follicular atresia (Payne and Runser, 1958; Louvet *et al.*, 1975; Scaramuzzi *et al.*, 1977; Hillier and Ross, 1979; Hillier *et al.*, 1979; Farookhi, 1981; Hillier, 1981; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Evans *et al.*, 1997). The death of granulosa cells may be caused by androgens interfering in production and/or action of estradiol (Bagnell *et al.*, 1982), or by direct actions of androgens on granulosa cells (Tetsuka *et al.*, 1995; Garrett and Guthrie, 1996). In cows, atresia is characterized by diminished granulosa cell aromatase enzyme activity (Mc Natty *et al.*, 1984a), and granulosa cells in atretic follicles do not express mRNA for aromatase ($P450_{arom}$ mRNA) (Xu *et al.*, 1995a). Therefore, diminished capacity of bovine granulosa cells to produce estradiol is associated with apoptosis of granulosa cells and follicular atresia. However, it is not known whether diminished capacity of granulosa cells to produce estradiol is a cause or an effect of granulosa cell apoptosis. In summary, androgens may be important regulators of estradiol production not only by providing substrate for estradiol synthesis, but also by regulating aromatase activity and apoptosis of granulosa cells. These regulatory events would mean that androgens exert a significant role in dominant follicle growth and atresia.

The present study will test the hypothesis that different types and amounts of androgens alter proliferation, viability, estradiol production, and aromatase enzyme activity *in vitro* of bovine granulosa cells isolated from the first-wave dominant follicle. To test this hypothesis, three experiments were conducted: **Experiment I** examined the effects of androgens on granulosa cell number (**Part A**), viability (**Part B**), and estradiol production (**Part C**). **Experiment II** determined if pre-treatment with different amounts of androgens alters subsequent capacity of granulosa cells to produce estradiol. **Experiment III** tested the effects of DHT, a non-aromatizable androgen, on production of estradiol by granulosa cells.

Materials and Methods

Reagents and hormones

The following items were obtained from Life Technologies, Inc. (Grand Island, NY): Endothelial-SFM growth medium with L-glutamine (SFM-growth), Endothelial-SFM plating medium with bovine vitronectin and L-glutamine (SFM-plating), penicillin G sodium, streptomycin sulfate, and trypan blue exclusion dye. 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), ethylenediamine tetraacetic acid (EDTA), and calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, monobasic phosphate, and dibasic phosphate were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Tris base was obtained from Boehringer Mannheim (Indianapolis, IN). TNE buffer (mM Tris, 2 M NaCl, 1mM EDTA, ph 7.4) and Hoechst 33258 dye were included in the FluoReporter Blue Fluorometric dsDNA Quantitation kit from Molecular Probes (Eugene, OR).

Tissue collection and bovine granulosa cell preparation

Ovaries from beef and dairy cows during Days 2-5 of the estrous cycle were collected from a slaughterhouse (MURCO, Plainwell, MI). Stage of the estrous cycle was determined by morphology of the corpus luteum (Ireland *et al.*, 1980). Pairs of ovaries from each cow were placed in individual bottles containing ice-cold phosphate buffered saline solution (0.01 M monobasic phosphate, 0.04 M dibasic phosphate, 0.1 M sodium chloride, 0.02 M EDTA; pH 7.4) and transported on ice to the laboratory within 4 h of slaughter. The largest follicle per pair of ovaries, which is the first-wave dominant follicle (Fortune, 1993), was dissected, ovarian stroma removed, and the follicle's diameter was recorded. Follicular fluid was aspirated from each follicle by puncturing it with a 22-gauge needle attached to a 3 ml syringe. The follicle shell was then bisected under sterile conditions and placed on a watch glass containing Endothelial-SFM growth medium (SFM-growth) supplemented with penicillin/streptomycin solution (1.5 mg/ml penicillin G sodium and 2.5 mg/ml streptomycin sulfate). Granulosa cells were gently scraped from the inner wall of each follicle into medium using a bent microspatula. Granulosa cells were pooled then washed in ice-cold medium and centrifuged (400 x g for 5 min at 4 °C) three times. Number of cells was estimated using a hemocytometer.

Androgens

The rationale to use of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), and dihydrotestosterone (DHT) is based on several previous observations. Each of the aforementioned types of androgens are in follicular fluid (Lacroix *et al.*, 1974; Mc Natty *et al.*, 1979b; Henderson *et al.*, 1982; Ireland and Roche, 1982; Dieleman *et al.*, 1983a; 1983b; Fortune and Hansel, 1985; Wise, 1987; Xu *et al.*, 1995a) and implicated in affecting follicular growth, atresia, estradiol production, and aromatase enzyme activity (Payne and Runser, 1958; Ohno *et al.*, 1973; Schwarzel *et al.*, 1973; Lacroxi *et al.*, 1974; Louvet *et al.*, 1975; Lucky *et al.*, 1977; Mori *et al.*, 1977; Scaramuzzi *et al.*, 1977; Kumari *et al.*, 1978; Leung *et al.*, 1978; Goff *et al.*, 1979; Henderson and Moon, 1979; Hillier and Ross, 1979; Hillier *et al.*, 1979; Peluso *et al.*, 1979; Daniel and Armstrong, 1980; Hillier *et al.*, 1980a; 1980b; Farookhi, 1981; Hillier and De Zwart, 1981; Hillier *et al.*, 1981a; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Welsh *et al.*, 1982; Dieleman et al., 1983a; Möstl *et al.*, 1984; Wise, 1987; Tonetta and di Zerega, 1989; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Bley *et al.*, 1997). Androstenedione (Dieleman *et al.*, 1983a), testosterone (Henderson and Moon, 1979), and DHEA (Lacroix *et al.*, 1974; Möstl *et al.*, 1984; Wise, 1987) have all been reported as the preferred substrate for ovarian estrogen synthesis in cattle. Because of this controversy, 19-OH-A, 19-OH-T, and DHEA were used in the present study to clarify which androgen is the preferred substrate for estradiol production by bovine granulosa cells. The androgen metabolites, 19-OH-A and 19-OH-T, rather than androstenedione and testosterone, were used because they are directly metabolized into estradiol (Figures 2 and 3). In contrast, androstenedione and testosterone can be interconverted or form other androgens such as DHT. In the present study, DHT was used because it is a non-aromatizable androgen that diminishes the activity of granulosa cell aromatase enzyme (Schwarzel *et al.*, 1973; Hillier *et al.*, 1979; 1980a) and causes atresia (Bagnell *et al.*, 1982).

Doses of androgens ranged from 10^{-8} M to 10^{-5} M. Except for 10^{-5} M, this range of concentrations of androgens spans the range of androgen concentrations in follicular fluid (Mc Natty *et al.*, 1979b; Henderson *et al.*, 1982; Ireland and Roche, 1982; Dieleman *et al.*, 1983a; 1983b; Fortune and Hansel, 1985; Wise, 1987; Xu *et al.*, 1995a).

Bovine granulosa cell culture

Except where noted, 10^5 bovine granulosa cells in 20 μl of SFM-growth medium were plated in 96-well Falcon Primaria culture plates (Becton Dickinson and Co., Lincoln Park, NJ) containing 180 μl of Endothelial-SFM plating medium (SFM-plating) previously equilibrated at 37°C. The media was supplemented with penicillin/streptomycin solution and different types and doses of androgens. Granulosa cells were incubated at 37°C in a humidified atmosphere (5 % CO_2 and 95 % air) for 2, 6, 12, or 18 h as indicated for each experiment. After incubation, cells were detached from culture wells by gently flushing them with the existing media in the wells, except where noted. Cells and/or media were collected from each well and placed in individual 1.5 ml Eppendorf tubes. If both cells and media were collected, Eppendorf tubes were centrifuged (400 x g for 10 min at 4°C) to form pellets of cells and media were removed carefully without disturbing the pellets and placed in individual Eppendorf tubes. Fresh, ice-cold SFM-growth medium (200 μl) supplemented with penicillin/streptomycin solution was then added to the cell pellet, and cells were resuspended and stored on ice until viability was assessed. If only media were collected, culture plates were centrifuged (400 x g for 1 min at 4°C) and approximately 140 μl of medium from each well was collected and placed in individual Eppendorf tubes. Culture plates containing cells were then stored at -80°C until assayed for DNA. Media were stored at -20°C until assayed for estradiol concentration.

Viability assessment

Viability of granulosa cells after isolation from follicles (initial) and viability after culture (final) were determined by mixing 200 μl of a 1:10 dilution of the initial cell suspension, or by mixing 200 μl of a cell suspension that had previously been stored on ice after completion of the culture period, with 200 μl of a 1:5 dilution of 0.4% trypan blue exclusion dye. The mixture was incubated at room temperature for 1 min. An aliquot was then placed on a hemocytometer and examined under the microscope. Stained cells were considered dead. Viability was expressed as percent of viable (non-stained) cells per total (stained + non-stained) cells in a well.

Estimation of DNA

Alterations in number of cultured granulosa cells were determined by estimation of granulosa cell DNA using the protocol provided in the FluroReporter Blue Fluorometric dsDNA Quantitation Kit. Briefly, culture plates containing cells that had previously been stored at -80°C were thawed at room temperature and 100 μl of double-distilled water was added per well. To lyse cells, the plate was incubated at 37°C for 1 h, frozen at -80°C, and then thawed at room temperature. Aqueous Hoechst 33258 dye in TNE buffer (25 μl Hoechst 33258 solution + 10 ml TNE buffer) was added to wells (100 μl / well) immediately before placing the plate on a microplate reader (CytoFluor 4000, PerSeptive Biosystems). Fluorescence was measured using excitation and emission filters centered at 360 nm and 460 nm, respectively. Background fluorescence readings for cell-free samples (100 μl dye + 60 μl SFM-plating medium + 100 μl double-distilled water) were subtracted from readings of samples containing cells. Amount of DNA in granulosa cells in each well was determined using a calf thymus DNA standard curve (Appendix A).

DNA standard curve

A stock solution of calf thymus DNA (6.7 μg ml^{-1} based on absorbance at 260 nm in a 1 cm path length cuvette, Molecular Probes, 1996) was prepared in TE buffer (10 mM Tris base, 1 mM EDTA, ph 7.4) and different concentrations (0 and 6.7 ng, 16.75 ng, 33.5 ng, 67 ng, 167.5 ng, 335 ng, 502.5 ng, and 670 ng per 100 μl) pipetted in triplicate into wells of a Falcon Primaria 96-well culture plate to construct a standard curve. Aqueous Hoechst 33258 dye in TNE buffer (25 μl Hoechst 33258 solution + 10 ml TNE buffer) was added to wells (100 μl / well) immediately before placing the plate on a microplate reader. Fluorescence was measured as stated above. Background fluorescence readings for DNA-free samples (100 μl dye + 100 μl TE buffer) were subtracted from readings of wells containing DNA.

Estradiol assay

Concentrations of estradiol in media were determined by radioimmunoassay using previously validated (Turzillo and Fortune, 1990; Ireland *et al.*, 1994), commercially available kits (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity of the assay was 0.5 pg/ml. Cross-reactivity of estradiol antiserum with estrone is 12.55 % and less than 1% with other steroids.

Experiments

Experiment I: Effects of types and amounts of androgens on proliferation, viability, and estradiol production

Part A. Cell number: To determine if androgens altered number of cells, granulosa cells were collected and processed for culture as described above. Cells were then plated in culture wells containing SFM-plating medium supplemented with different doses of 19-OH-A, 19-OH-T, DHEA, DHT, or no androgen, as explained earlier. At the end of the 18 h culture, media were collected, and culture wells containing cells were assayed for DNA.

Part B. Viability: To determine if androgens altered viability, initial viability of granulosa cells after isolation from follicles was compared with viability after the 18 h culture period in medium containing different types and amounts of androgens. Granulosa cells were collected, processed, and cultured as described in Part A.

Part C. Estradiol production: To determine if androgens altered estradiol production, media from the granulosa cells in Part B were collected after the 18 h culture period and analyzed for concentration of estradiol.

To test whether the various types of androgens were converted into estradiol at different rates, granulosa cells were isolated and processed for culture as previously stated, except cells were plated into culture wells containing medium supplemented with 10^{-6} M of either 19-OH-A, 19-OH-T, or DHEA and cultured for 1, 3, 6, 12, or 18 h. Media were collected at the end of each culture period and assayed for estradiol.

Experiment II: Effects of types and amounts of androgen pre-treatments on viability and aromatase enzyme activity

To evaluate the effects of androgen pre-treatments on subsequent capacity of granulosa cells to produce estradiol, residual steroids in media after androgen priming had to be removed without cell loss before final treatments began. To determine if granulosa cells could be washed in culture wells to remove residual steroids without cell loss, granulosa cells (5×10^5 cells in 50 μl of SFM-growth medium) were plated in culture wells containing 150 μl of SFM-plating medium and cultured for 1 h. Cells in each well were then washed 0, 3, 4, or 5 times by centrifuging the culture plate (400 x g for 1 min at 4 °C), removing 140 μl of medium from each well, and then replacing it with fresh, ice-cold SFM-growth medium. Medium was not replaced after the final wash. In order to evaluate cell loss, DNA content per culture well was determined after each wash.

To determine if washes removed residual steroids after androgen priming, granulosa cells were pre-treated with different types and amounts of androgens for 6 h, washed five times as stated above. Estradiol concentration was then measured in media from the final wash as an index for removal of all steroids in media.

To determine effects of androgens on aromatase enzyme activity, granulosa cells were processed for culture as explained above and pre-treated with different types (19-OH-A, 19-OH-T, DHEA, DHT, and no androgen) and amounts (10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M) of androgens for 6 h. Cells were then washed five times to remove residual steroids and cultured for an additional 12 h with or without 10^{-6} M 19-OH-A. Estradiol concentration was then measured in media. To determine if the various androgen pre-treatments affected viability, granulosa cell viability after the 18 h culture period was compared among pre-treatments, and with the initial viability of granulosa cells after isolation from follicles.

Experiment III: Effect of DHT on production of estradiol by bovine granulosa cells

To determine the effects of DHT on capacity of granulosa cells to produce estradiol, granulosa cells were cultured in medium containing 19-OH-A ($1.01\text{x}10^{-6}$ or $2\text{x}10^{-6}$ M) or 19-OH-A (10^{-6} M) in combination with DHT (10^{-8} or 10^{-6} M). The $1.01\text{x}10^{-6}$ and $2\text{x}10^{-6}$ M doses of 19-OH-A were used to expose cells in the control groups to the same total concentration of androgen as the cells treated with combinations of DHT and 19-OH-A. Media were collected and concentration of estradiol in media was determined. Granulosa cells were isolated, processed, and cultured as previously stated.

Statistical analysis

All analyses were performed using SPSS Base 8.0 (SPSS Inc., Chicago, IL). Experimental data are expressed as arithmetic means (± SEM) of measurements of triplicate or more culture wells or aliquots. Experiments were replicated at least twice with different pools of bovine granulosa cells isolated from ovaries obtained during separate trips to an abattoir with the following exceptions: Experiment I Part C (conversion rates of androgens into estradiol), Experiment II's washing 0 to 5 times to remove residual steroids, and Experiment III. Unless stated otherwise, significance was $P<0.05$. Except where indicated, the effects described are representative of results obtained in separate replicates, although the absolute levels of estradiol production and the magnitude of response to androgen treatments varied between different pools of cells.

Two-way analysis of variance (ANOVA) was used to test the effects of type and dose of androgen on alterations in granulosa cell DNA (Experiment I Part A), viability (Experiment I Part B), and estradiol production (Experiment I Part C). When a significant difference was found, the Bonferroni t-test was used to determine which means differed significantly. In Experiment I Part B, linear regression analysis was used to determine if a linear relationship existed between androgen dose and granulosa cell viability. In Experiment I Part C, linear regression analysis was used to determine slopes for each type of androgen for each experimental replicate, which are displayed in Table 1. Differences between slopes for each type of androgen for each replicate and differences between replicates for each androgen were determined by the equivalent t-test for slope using the equation $|t| = (\beta_{1A} - \beta_{1B}) \ / \ s\{\beta_{1A}\}$. β_{1A} and β_{1B} are the slopes of different androgens A and B, respectively, and $s\{\beta_{1A}\}$ is the standard deviation of the slope for androgen A (Neter *et al.*, 1990; Frenud and Wilson, 1993).

In Experiment II, one-way ANOVA was used to test the effect of washing granulosa cells on alterations in granulosa cell DNA. Two-way ANOVA was used to determine if androgen pre-treatments altered subsequent estradiol production in Experiment II. When a significant difference was found, the Bonferroni t-test was used to determine which means differed significantly. Linear regression analysis was then used to determine if the subsequent capacity of granulosa cells to produce estradiol increased linearly for each androgen pre-treatment.

In Experiment III, one-way ANOVA was used to test the effect of DHT on estradiol production.

Results

Experiment I: Effects of types and amounts of androgens on proliferation, viability, and estradiol production

Part A. Cell number: Treatment of granulosa cells with androgens did not alter ($P>0.10$) amount of granulosa cell DNA per well (data not shown).

Part B. Viability: Viability of granulosa cells for the four replicates were similar, thus data were pooled. The final viability for granulosa cells cultured in 10^{-6} M and 10^{-5} M 19-OH-A was the same ($P>0.1$) as initial viability, whereas final viabilities for cells cultured in all other androgen treatments were lower ($P<0.05$) than initial viability (Figure 11). Viability was higher ($P<0.05$) for granulosa cells cultured in 19-OH-A, 19-OH-T, or DHEA compared with cells in media without androgen (NA) or DHT. Overall viability of granulosa cells increased ($P<0.05$) with increasing dose of 19-OH-A.

Part C. Estradiol production: Granulosa cells treated with DHT or receiving no androgen produced negligible amounts of estradiol (Figure 12). For each replicate, cells treated with 19-OH-A or 19-OH-T consistently produced more ($P<0.05$) estradiol compared with cells treated with DHEA at the 10^{-6} M and 10^{-5} M doses, but not at lower doses. Since amounts of estradiol produced for each different androgen varied greatly ($P<0.05$) among replicates, pooling of results was precluded. However, despite the marked difference in response to androgens among replicates, the linear increase in estradiol production was consistently greater ($P<0.05$) with 19-OH-A and 19-OH-T treatments compared with DHEA or DHT (Table 1). Note, the linear increase in estradiol was greater ($P<0.05$) for 19-OH-T compared with 19-OH-A and for DHEA compared with DHT for three replicates (Table 1).

Accumulation of estradiol in media was similar at each time point for granulosa cells treated with 19-OH-A or 19-OH-T, but greater ($P<0.05$) for each of these androgens compared with DHEA (Figure 13).

Experiment II: Effects of types and amounts of androgen pre-treatments on viability and aromatase enzyme activity

Cells could be washed five times without ($P>0.10$) alterations in amount of granulosa cell DNA per culture well (data not shown). After five washes, residual estradiol in media of granulosa cells pre-treated with androgens ranged from 0 to 0.19 ng/ml.

Final viability of granulosa cells differed ($P<0.05$) for the replicates which precluded pooling of results. For each replicate, final viability of cells was lower ($P<0.05$) than initial viability (Figure 14) at each androgen pre-treatment dose except for cells pre-treated with 10^{-5} M 19-OH-A or 10^{-8} M 19-OH-T, which was similar ($P>0.1$) to initial viability. In each replicate, viability was higher ($P<0.05$) at each dose, except 10^{-8} M (in Panel A), for cells pre-treated with 19-OH-A or 19-OH-T compared with DHEA, DHT, or no androgen.

Amount of estradiol produced during culture varied ($P<0.05$) among the two studies, which precluded pooling of results. Capacity of granulosa cells to convert 19-OH-A into estradiol increased ($P<0.05$) linearly with increasing pre-treatment dose of 19-OH-A, 19-OH-T, and DHEA, but not DHT, for each replicate (Figure14). Cells pre-treated with 19-OH-A and 19-OH-T consistently produced more ($P<0.05$) estradiol compared with cells pre-treated with DHEA or DHT at the 10^{-6} M and 10^{-5} M doses, but not at lower doses.

Experiment III: Effect of DHT on production of estradiol by bovine granulosa cells

Each dose of DHT diminished ($P<0.05$) capacity of granulosa cells to convert 19-OH-A into estradiol compared with controls without DHT (Figure 15).

Figure 11. Effect of androgen treatments on viability of bovine granulosa cells.

Pairs of ovaries from cows during Days 2-5 of the estrous cycle were collected from a slaughterhouse. The largest follicle per pair of ovaries, which is the first-wave dominant follicle (Fortune, 1993), was isolated. Follicular fluid was aspirated from the follicle, and the follicle was cut in half and placed on a watch glass containing Endothelial-SFM growth medium (SFM-growth). Granulosa cells were gently scraped into medium. Cells were plated in a 96-well culture plate (10^5 cells / well) containing Endothelial-SFM plating medium (SFM-plating) supplemented with different concentrations (x-axis) of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), or no androgen (NA). Cells were incubated for 18 h. Media and cells were then collected and separated as explained in Methods. Initial and final viability were assessed using trypan blue exclusion dye. Results were similar for 4 separate experiments, thus data were pooled for final statistical analysis. Values are overall means (± SEM) of 4 separate experiments. Each bar depicts mean values for 12 replicates.

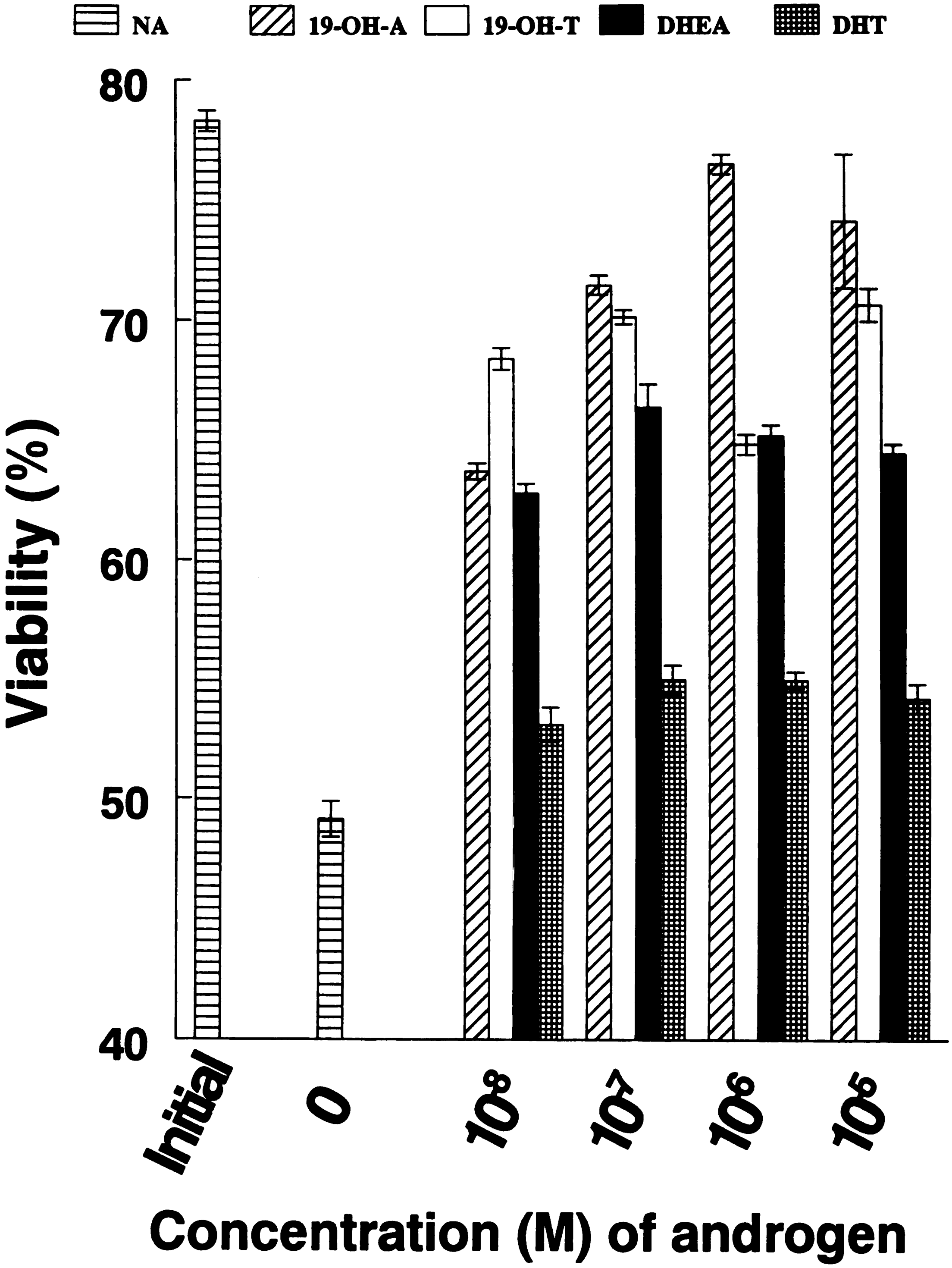
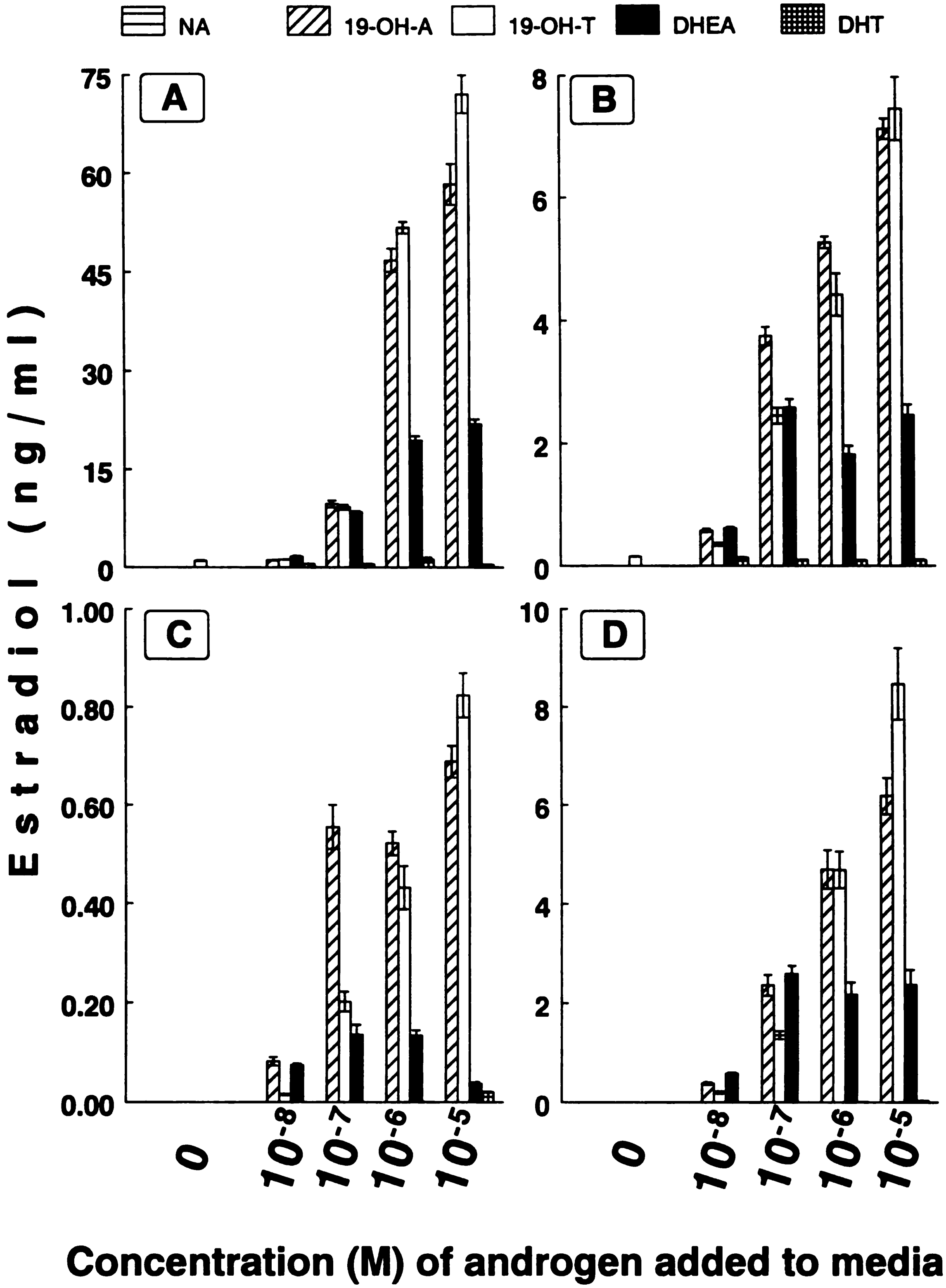

NA
19-OH-A
19-OH-T
DHEA
DHT
Viability (%)
80
70
60
50
40
Initial
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration (M) of androgen

Figure 12. Effect of androgen treatments on estradiol production by bovine granulosa cells. Granulosa cells were isolated and processed for culture as explained in Figure 11's legend. Cells were plated in 96-well culture plates (10^5 cells / well) containing Endothelial-SFM plating medium supplemented with different concentrations (x-axis) of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), or no androgen (NA). Cells were incubated for 18 h. Media and cells were collected and separated as explained in Methods. Radioimmunoassay was used to determine concentration of estradiol in media. Each bar depicts mean (± SEM) values for 6 replicates. Each panel (A, B, C, and D) shows results of four separate experiments.



NA
19-OH-A
19-OH-T
DHEA
DHT
A
B
C
D
Estradiol (ng/ml)
75
60
45
30
15
0
8
6
4
2
0
1.00
0.80
0.60
0.40
0.20
0.00
10
8
6
4
2
0
0
10-8
10-7
10-6
10-5
Concentration (M) of androgen added to media

Table 1 - Linear regression analysis of the effects of different types[1] and doses[2] of androgens on amount of estradiol produced during cell culture[3].

	19-OH-A[1]	19-OH-T[1]	DHEA[1]	DHT[1]
Experiment A	20.83*$^{a\,A}$	25.48*$^{b\,A}$	7.14*$^{c\,A}$	0.04 $^{d\,A}$
B	2.29*$^{a\,B}$	2.35*$^{a\,B}$	0.48*$^{b\,B}$	- 0.03 $^{c\,A}$
C	0.18*$^{a\,C}$	0.27*$^{b\,C}$	0.01 $^{c\,C}$	0.01*$^{c\,A}$
D	1.91*$^{a\,D}$	2.81*$^{b\,D}$	0.50*$^{c\,B}$	0.01*$^{d\,A}$

[1] 19-OH-A= 19-hydroxy-androstenedione, 19-OH-T= 19-hydroxy-testosterone, DHEA= dehydroepiandrosterone, DHT= dihydrotestosterone.

[2] Androgen doses (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) used to calculate slopes (b).

[3] Data shown in Figure 12.

*b = $P<0.01$

[a] Slopes with dissimilar superscripts in a row are statistically different ($P<0.05$).

[A] Slopes with dissimilar superscripts in a column are statistically different ($P<0.05$).

Figure 13. Effect of different androgen substrates on production of estradiol.

Granulosa cells were isolated and processed for culture as explained in Figure 11's legend. Cells were plated in 96-well culture plates (10^5 cells / well) containing Endothelial-SFM plating medium supplemented with 1 x10^{-6} M of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), or dehydroepiandrosterone (DHEA). Cells were incubated for 1, 3, 6, 12, or 18 h (x-axis). Media and cells were then collected and separated as explained in Methods. Radioimmunoassay was used to determine concentration of estradiol that accumulated in media (y-axis). Each bar depicts mean (± SEM) values for 6 replicates from a single experiment.

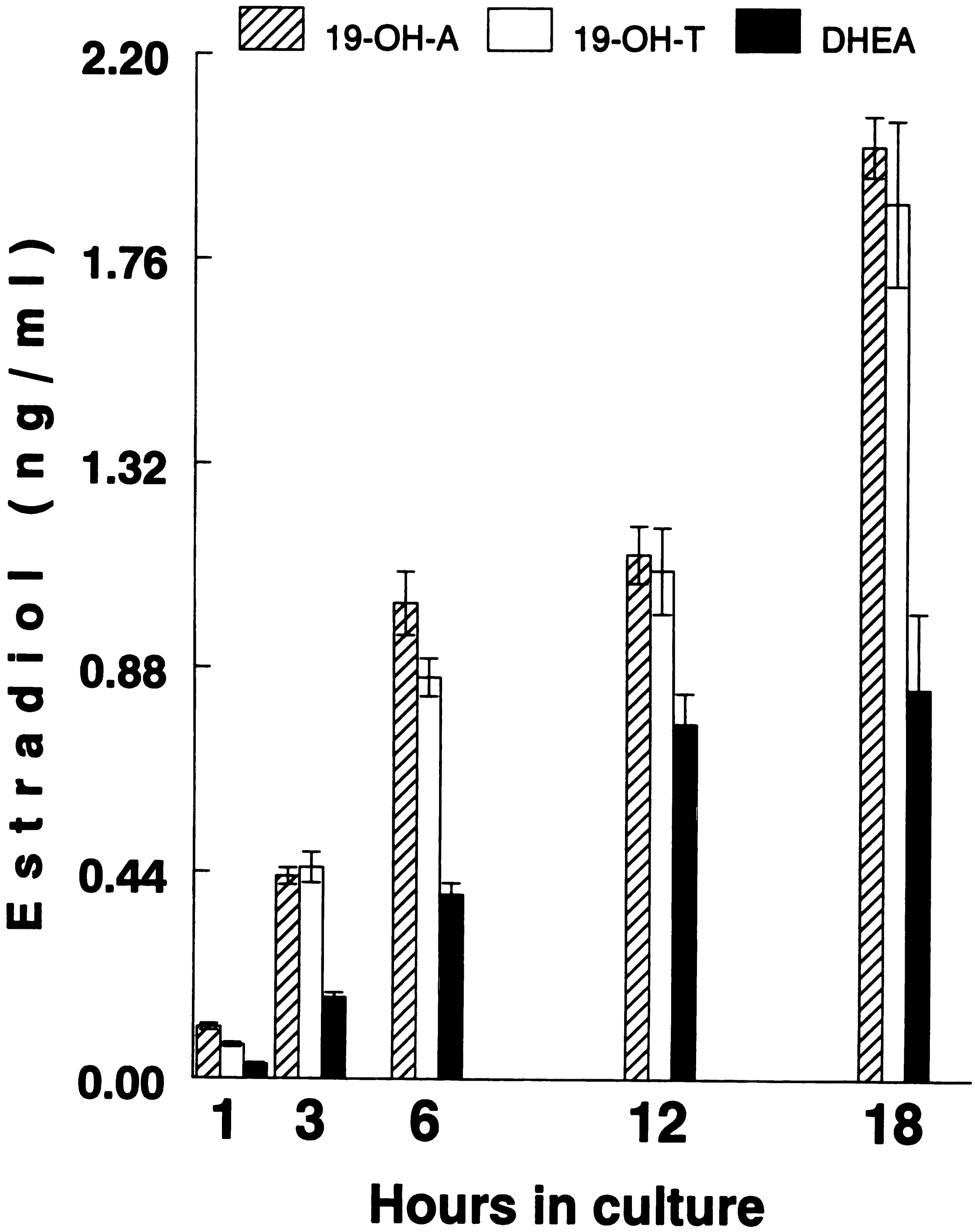

19-OH-A
19-OH-T
DHEA
Estradiol (ng/ml)
2.20
1.76
1.32
0.88
0.44
0.00
1
3
6
12
18
Hours in culture

Figure 14. Effects of pre-treatment of bovine granulosa cells with different types and doses of androgens on aromatase activity. Granulosa cells were isolated and processed for culture as explained in Figure 11's legend. Cells were plated in 96-well culture plates (10^5 cells / well) containing Endothelial-SFM plating medium supplemented with different concentrations (x-axis) of 19-hydroxy-androstenedione (19-OH-A), 19-hydroxy-testosterone (19-OH-T), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), or no androgen (NA). Cells were incubated for 6 h. Media was then collected and cells were washed as explained in Methods. Fresh Endothelial-SFM plating medium (140 μl) supplemented with either 10^{-6} M 19-OH-A or no androgen (Control) was added to cells. Cells were incubated for 12 h. Media and cells were then collected and separated as explained in Methods. Initial and final viability (Panels A and C) were assessed using trypan blue exclusion dye. Radioimmunoassay was used to determine concentration of estradiol in media (Panels B and D). Each bar depicts mean (± SEM) values for 3 (Panels A and C) or 6 (Panels B and D) replicates. Panels A and B, and C and D represent values for two separate experiments, respectively.

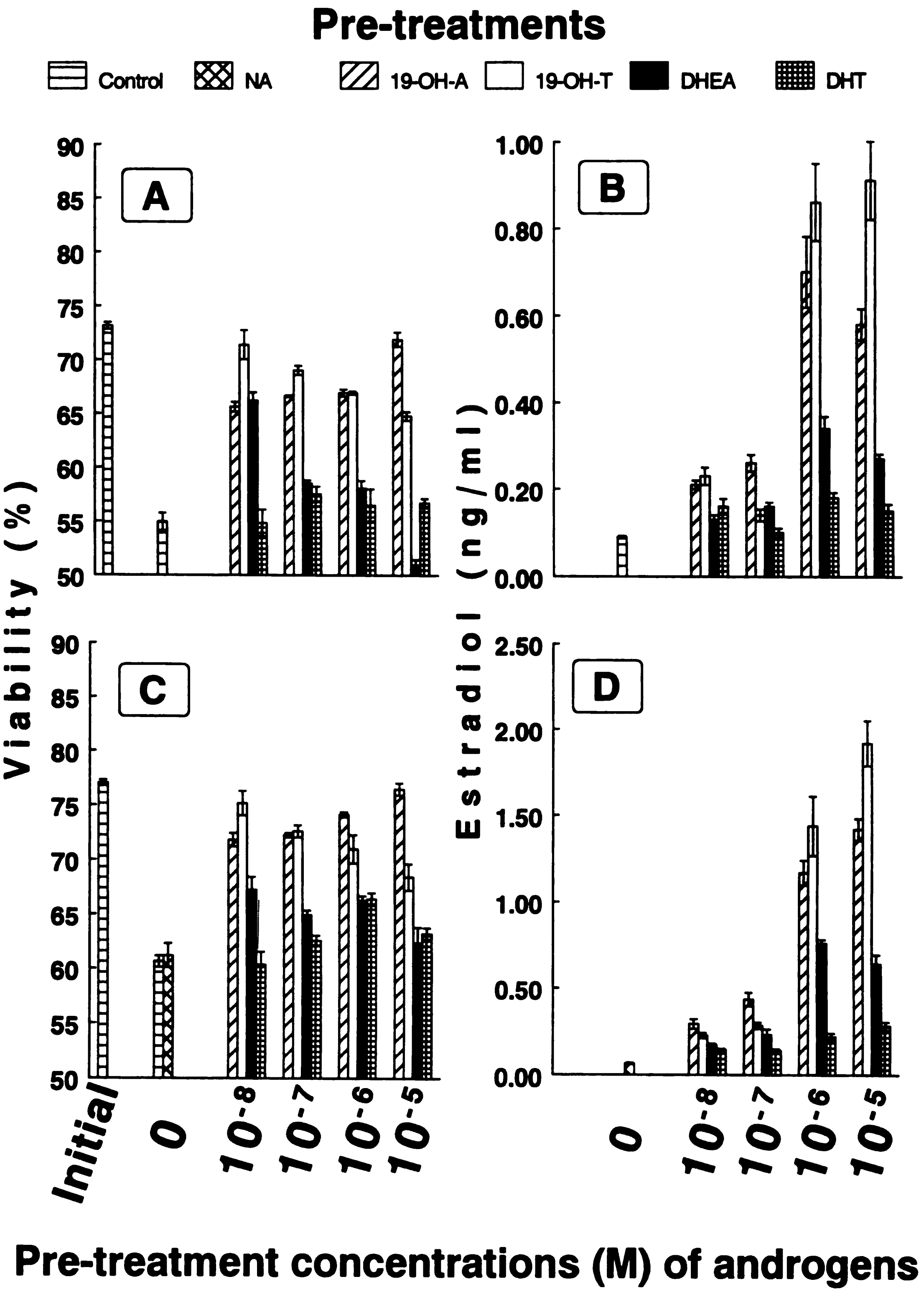
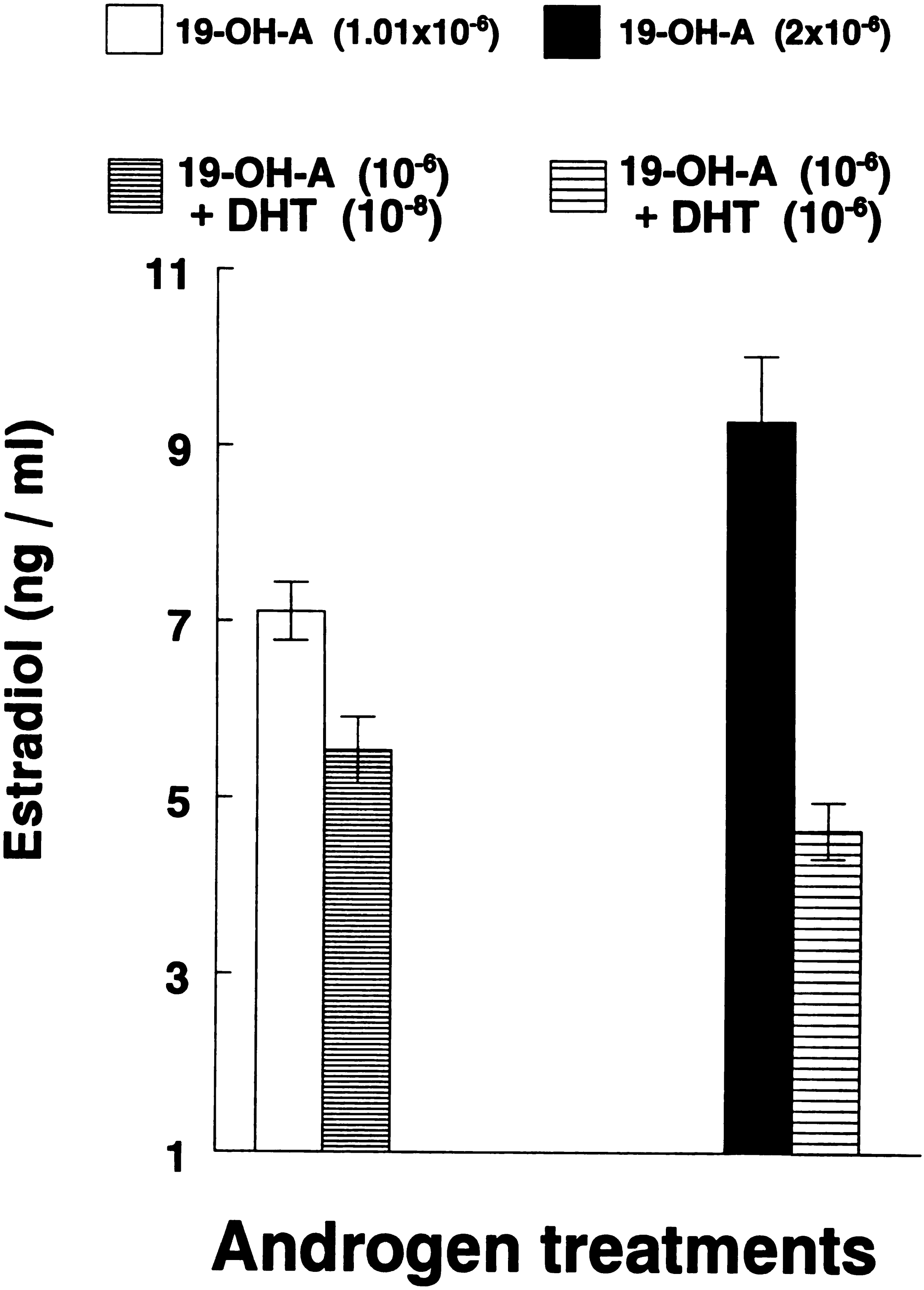

Pre-treatments
Control
NA
19-OH-A
19-OH-T
DHEA
DHT
A
B
C
D
Viability (%)
Estradiol (ng/ml)
90
85
80
75
70
65
60
55
50
1.00
0.80
0.60
0.40
0.20
0.00
2.50
2.00
1.50
1.00
0.50
0.00
Initial
0
10-8
10-7
10-6
10-5
Pre-treatment concentrations (M) of androgens

Figure 15. Effect of dihydrotestosterone on the capacity of bovine granulosa cells to convert 19-hydroxy-androstenedione into estradiol. Granulosa cells were isolated and processed for culture as explained in Figure 11's legend. Cells were plated in 96-well culture plates (10^5 cells / well) containing Endothelial-SFM plating medium supplemented with different concentrations (legend) of 19-hydroxy-androstenedione (19-OH-A) or 19-OH-A in combination with dihydrotestosterone (DHT). Cells were incubated for 6 h. Media was then collected. Radioimmunoassay was used to determine concentration of estradiol in media. Each bar depicts mean (± SEM) values for 3 replicates for a single experiment.


19-OH-A (1.01x10-6)
19-OH-A (2x10-6)
19-OH-A (10-6) + DHT (10-8)
19-OH-A (10-6) + DHT (10-6)
Estradiol (ng / ml)
11
9
7
5
3
1
Androgen treatments

Discussion

The most significant findings of the present *in vitro* study are that different types and amounts of androgens did not alter proliferation, but did alter the viability, estradiol production, and aromatase enzyme activity of granulosa cells isolated from the first-wave dominant follicle.

The estradiol produced by granulosa cells treated with 19-OH-A, 19-OH-T, or DHEA in the present study did not increase number of granulosa cells during the *in vitro* culture period. This finding is in contrast to results of previous studies showing positive effects of estradiol on granulosa cell proliferation in rats *in vivo* (Williams, 1940; Goldenberg *et al.*, 1972; Tonetta and Ireland, 1984) and *in vitro* (Hillier, 1981). In speculation, the presence of androgens and/or production of other unknown factors during the *in vitro* culture period may have inhibited the mitogenic effect of estradiol in the present study. Alternatively, the culture period may have been insufficient for androgens or estradiol to stimulate cell proliferation.

Different types of androgens affected viability of granulosa cells *in vitro* in several different ways. First, viability decreased if granulosa cells were cultured without androgen. Second, viability of granulosa cells treated with DHT was consistently higher than viability of cells cultured without androgen. Third, viability was enhanced when granulosa cells were treated with 19-OH-A, 19-OH-T, or DHEA compared with DHT or no androgen. While the reason for these different responses is unknown, this finding may indicate that several sub-populations of granulosa cells within a first-wave dominant follicle have different steroid requirements for survival. For example, one sub-population of granulosa cells may not require androgen or estradiol to survive since approximately

50% of the cells cultured without androgen remained viable after the culture period. In contrast, a small sub-population of granulosa cells may be androgen responsive since cells treated with DHT, a non-aromatizable androgen, had a higher viability compared with cells treated with no androgen. Finally, a third sub-population of granulosa cells may require aromatizable androgen and/or estradiol to survive. The existence of sub-populations of granulosa cells that have different steroid requirements for survival may also indicate that sub-populations of granulosa cells within a follicle have different functional capabilities. Indeed, ovarian follicles are composed of small and large granulosa cells, and the large cells are steroidogenic (Rao *et al.*, 1991; Lederer *et al.*, 1993; Sanbuissho *et al.*, 1993). Furthermore, bovine granulosa cells near the antrum of a follicle have a greater capacity to produce steroids than mural granulosa cells (Roberts and Echternkamp, 1994).

Granulosa cells die through the process of apoptosis (Hughes and Gorospe, 1991; Tilly *et al.*, 1991; Luciano *et al.*, 1994). Therefore, alterations in viability of granulosa cells, as measured by trypan blue exclusion dye, may provide insight into the role androgens have in apoptosis. Androgens are implicated in causing follicular atresia (Payne and Runser, 1958; Louvet *et al.*, 1975; Scaramuzzi *et al.*, 1977; Hillier and Ross, 1979; Hillier *et al.*, 1979; Farookhi, 1981; Hillier, 1981; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Evans *et al.*, 1997) and follicular atresia is initiated with the death of granulosa cells (Luciano *et al.*, 1994; Peluso *et al.*, 1996). In contrast, estradiol is anti-atretogenic (Payne and Hellbaum, 1955; Hillier, 1981; Billing *et al.*, 1993; Luciano *et al.*, 1994). Taken together, these findings could explain why granulosa cells treated with androgen substrates, which are converted to estradiol, are more viable than those treated with DHT or no androgen. Alternatively, androgens may block the action of estradiol. For example, in a study conducted in rats by Bagnell *et al.* (1982), DHT injections trigger follicular atresia, but estradiol co-injected with DHT prevents the atresia. The reason DHT induced atresia may be because DHT is non-aromatizable and it competitively inhibits granulosa cell aromatase activity in rats (Hillier and Ross, 1979; Hillier *et al.*, 1979; Hillier *et al.*, 1980a). This could also explain why granulosa cells pre-treated with DHT in the present study produced less estradiol and were less viable than granulosa cells pre-treated with aromatizable androgen.

Different types of androgens were converted differentially into estradiol during culture of bovine granulosa cells isolated from the first-wave dominant follicle. At the two lowest doses of 19-OH-A, 19-OH-T, and DHEA tested there was no consistent evidence that granulosa cells treated with any of these androgens produced significantly more estradiol. However, limited availability of substrate may explain this finding. Based on accumulation of estradiol in culture media over time, DHEA is converted by granulosa cells into estradiol at a slower rate than 19-OH-A or 19-OH-T. A possible reason for this may be because 19-OH-A and 19-OH-T require fewer reaction steps in the transition into estradiol compared with DHEA (Figure 3; Hansel and Convey, 1983; Steinkampf *et al.*, 1987; Gore-Langton and Armstrong, 1994, Xu *et al.*, 1995a; Conley and Bird, 1997). Also, 19-OH-A and 19-OH-T are committed to become estradiol, whereas DHEA must first be converted into androstenedione (Figure 3; Rubin *et al.*, 1963; Zelezink *et al.*, 1974; Madej, 1980; Jones *et al.*, 1983; Gore-Langton and Armstrong, 1994) prior to being converted into estradiol or other androgens (Figure 3;

Lacroix *et al.*, 1974; Hansel and Convey, 1983; Mc Natty *et al.*, 1984b, Richards *et al.*, 1987; Payne *et al.*, 1992; Turillo and Fortune, 1992; Gore-Langton and Armstrong, 1994; Xu *et al.*, 1995a; Conley and Bird, 1997). While the findings of the present study did not establish whether androstenedione or testosterone was the preferred substrate for estradiol synthesis by bovine granulosa cells, the results from Experiment I implied that 19-OH-A and 19-OH-T, metabolites of androstenedione and testosterone, were better converted into estradiol than DHEA. Although, at the lower doses of androgen there was no clear substrate preference, granulosa cells treated with high doses of 19-OH-A and 19-OH-T consistently produced more estradiol than those with DHEA. In fact, at the highest physiological dose (10^{-6} M), 19-OH-A and 19-OH-T were equally converted into estradiol. However, at the pharmacological dose (10^{-5} M) granulosa cells treated with 19-OH-T produced more estradiol than those treated with 19-OH-A. There is no apparent explanation for this finding. Taken together these results clearly show that estradiol production by granulosa cells is effected by type and amount of androgen substrate. Hence, the higher estradiol produced by the first-wave dominant follicle compared to the subordinate follicles (Padmanabhan *et al.*, 1984; Ireland and Roche, 1987; Fortune, 1994) may be attributed to enhanced production of androstenedione and/or testosterone than DHEA or DHT by theca cells compared with theca cells from subordinate follicles.

The first-wave dominant follicle develops during Days 2 to 3 of the estrous cycle and persists approximately until Day 10 (Figure 1; Ireland and Roche, 1983b; Evans *et al.*, 1997). However, estradiol production by the dominant follicle is transient peaking around Days 3 to 4 (Figure 1; Ireland and Roche, 1983 b; Evans *et al.*, 1997), implying that aromatase enzyme activity is also transient. Thus, the reason for the marked variability in estradiol production for the different pools of granulosa cells in the present study is probably best explained by the transient nature of the aromatase enzyme in granulosa cells of the first-wave dominant follicle, coupled with the absence of a more reliable method to precisely define when aromatase activity is at its peak in a dominant follicle. Nevertheless, despite the marked differences in aromatase enzyme activity in granulosa cells of the first-wave dominant follicle *in vitro*, cell viability was similar. This finding indicates that a decrease in capacity of dominant follicles to produce estradiol may precede loss in granulosa cell viability, or that capacity of granulosa cells to produce estradiol is unrelated to their viability. However, the latter seems unlikely since aromatase enzyme activity is decreased in atretic dominant follicles (Ireland and Roche, 1983a; Mc Natty *et al.*, 1984a). In future studies, it will be important to establish the relationship of the activity of the aromatase enzyme system with initiation of apoptosis in bovine granulosa cells.

My previous studies showed that incubation of granulosa cells from the first-wave dominant follicle with different types and amounts of androgens for 18 h affected granulosa cell viability and that there was differential conversion of the different types of androgens into estradiol. The final studies for this thesis examined whether pre-treatment of granulosa cells for 6 h with different types and amounts of androgens affected subsequent capacity to produce estradiol, and if co-treatment of granulosa cells with aromatizable and non-aromatizable affected estradiol production. Pre-treatment of granulosa cells with 19-OH-A or 19-OH-T enhanced viability and subsequent aromatase enzyme activity compared to that of granulosa cells pre-treated with DHEA, DHT, or no androgen. This finding implies that not only are androgen substrates important for estradiol production, but they also positively affect health and aromatase enzyme activity of granulosa cells. The reason granulosa cells pre-treated with 19-OH-A or 19-OH-T were more viable after the culture period compared with cells pre-treated with DHEA, DHT, or no androgen is not certain, but may, in part, be due to the enhanced amounts of estradiol produced during the 6 h pre-treatment period. As mentioned earlier, estradiol may protect granulosa cells from the harmful effects of androgens. In addition, estradiol enhances aromatase enzyme activity (Daniel and Armstrong, 1983) which may explain why pre-treatment of granulosa cells with 19-OH-A or 19-OH-T enhanced subsequent aromatase enzyme activity compared with cells receiving DHEA, DHT, or no androgen. Alternatively, androgens may directly enhance aromatase enzyme activity (Lucky *et al.*, 1977; Daniel and Armstrong, 1980; Hillier and De Zwart, 1981; Welsh *et al.*, 1982). In contrast, granulosa cells pre-treated with DHT in the present study had the lowest subsequent aromatase enzyme activity, perhaps because DHT pre-treatment lowered granulosa cell viability. In addition, DHT may be a competitive inhibitor of the aromatase enzyme system in cows, like it is in rats (Hillier *et al.*, 1979; Hillier *et al.*, 1980a) and humans (Schwarzel et al., 1973). If so, this explains why DHT diminished the capacity of bovine granulosa cells to convert 19-OH-A into estradiol in Experiment III. Alternatively, since final viability of granulosa cells in Experiment III was not measured, the possibility that the diminished capacity of granulosa cells to convert 19-OH-A into estradiol was caused by their lower viability cannot be ruled out.

Results from the present study provide insight into how androgens could regulate the life span of the first-wave dominant follicle. For example, the dominant follicle on Days 2 to 5 of the estrous cycle is growing while serum concentrations of FSH are high and pulsatile secretion of luteinizing hormone (LH) is low (Figure 1; Ireland and Roche, 1983b; Evans *et al.*, 1997). The higher FSH not only maintains granulosa cell aromatase enzyme activity (Zeleznik *et al.*, 1974; Moon *et al.*, 1975; Steinkampf *et al.*, 1987) and estradiol production, but also inhibits 5α-reductase enzyme activity and production of 5α-reduced steroids, such as DHT (Payne *et al.*, 1992). The low level of LH is sufficient to stimulate production of aromatizable androgens for estradiol production (Mc Natty *et al.*, 1984a; Gore-Langton and Armstrong, 1994). Relatively high intrafollicular concentrations of estradiol protect granulosa cells in the dominant follicle from androgens (Payne and Hellbaum, 1955; Scaramuzzi *et al.*, 1977; Hillier, 1981; Bagnell *et al.*, 1982; Billing *et al.*, 1993). However, as FSH decreases during Days 2 to 5 of the estrous cycle, the ratio of non-aromatizable to aromatizable androgens produced by theca cells of the dominant follicle could increase resulting in a reduction in aromatase activity and estradiol production. Whether the intrafollicular ratio or non-aromatizable to aromatizable androgens is increased during development of the first-wave dominant follicle is unknown. An increase in non-aromatizable androgens, such as DHT, would be expected to decrease the intrafollicular amount of estradiol available to protect granulosa cells from the increasing amounts of androgens (Louvet *et al.*, 1975; Hillier *et al.*, 1979; Farookhi, 1981; Terranova and Greenwald, 1981; Mc Natty *et al.*, 1984a; Billing *et al.*, 1993) triggered by the increase in episodic secretion of LH (Figure 1). A high intrafollicular concentration of non-aromatizable androgens, such as DHT, towards the end of the growth phase of the dominant follicle is associated with loss of LH receptors on granulosa cells (Evans *et al.*, 1997). Together, the reduction of estradiol production, coupled with the increased intrafollicular amounts of non-aromatizable androgens, and loss of LH receptors, leads to granulosa cell apoptosis (Luciano *et al.*, 1994; Peluso *et al.*, 1996), which eventually causes the dominant follicle to become atretic (Payne and Runser, 1958; Louvet *et al.*, 1975; Hillier and Ross, 1979; Hillier *et al.*, 1979; Scaramuzzi *et al.*, 1977; Farookhi, 1981; Hillier, 1981; Terranova and Greenwald, 1981; Bagnell *et al.*, 1982; Billing *et al.*, 1993; Gore-Langton and Armstrong, 1994; Evans *et al.*, 1997). Hence, alterations in intrafollicular levels of aromatizable and non-aromatizable androgens may alter granulosa cell aromatase activity, estradiol production, and viability, and by doing so, determine the physiological fate of a dominant follicle. However, whether the ratio of aromatizable to non-aromatizable androgens is altered during selection, dominance, and atresia of dominant follicles is unknown for any species.

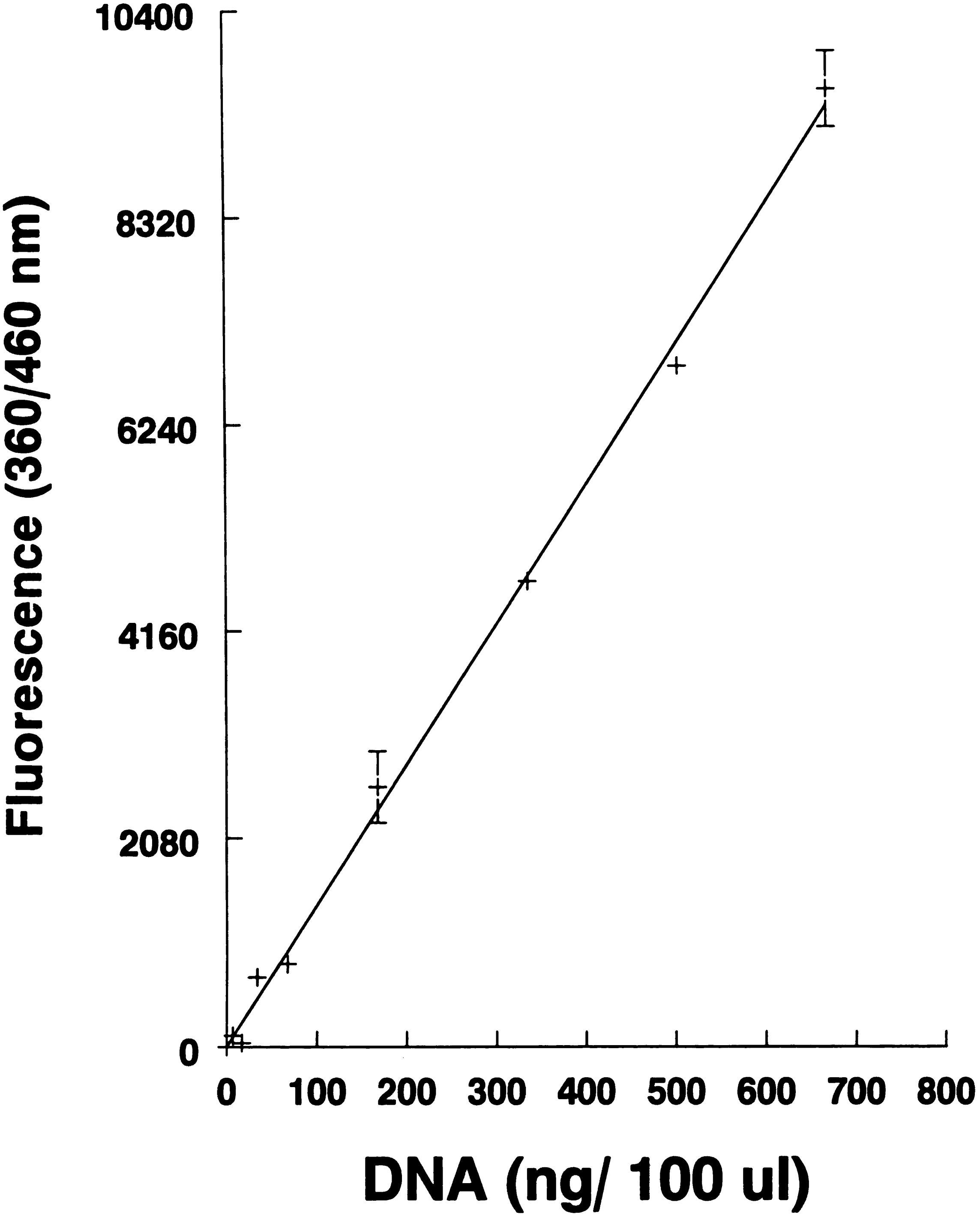
Summary and Conclusions

Treatment of bovine granulosa cells from the first-wave dominant follicle with different types and doses of androgens *in vitro* resulted in the following: a) no effect on cell number; b) enhanced viability of cells treated with 19-OH-A or 19-OH-T compared with cells treated with DHT or no androgen; c) higher estradiol production by cells treated with 19-OH-A or 19-OH-T than with DHEA, DHT, or no androgen; d) similar amounts of estradiol accumulation in media for cells treated with 19-OH-A or 19-OH-T; e) enhanced aromatase enzyme activity in cells pre-treated with 19-OH-A or 19-OH-T compared with DHEA, DHT, or no androgen; and f) diminished capacity of DHT treated granulosa cells to convert 19-OH-A into estradiol. These findings lead to the conclusion that type and amount of androgen alter viability, estradiol production, and aromatase activity of granulosa cells isolated from the first-wave dominant follicle. Thus, regulation of androgen synthesis appears to have an important regulatory role in dominant follicle growth, differentiation, and atresia.

APPENDIX A

Figure 16. Relationship of amount of calf thymus DNA to fluorescence.

Serial dilutions of a calf thymus DNA (6.7 μg / ml) solution in TE buffer were prepared and pipetted into wells of a 96-well culture plate (x-axis). Aqueous Hoechst 33258 dye in TNE buffer was added to each well and fluorescence (y-axis) was measured in a microplate reader using excitation and emission filters centered at 360 nm and 460 nm respectively. Fluorescence of DNA was determined by subtracting background fluorescence for wells containing only dye and TE buffer. Each symbol depicts mean (± SEM) values for a total 5 replicates form 2 separate plates.



10400
8320
6240
4160
2080
0
0
100
200
300
400
500
600
700
800
Fluorescence (360/460 nm)
DNA (ng/ 100 ul)

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