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Disruption of cytokeratin 18-containing intermediate filaments in bovine luteal cells: Effects on Fas expression, progesterone secretion, and FasL-induced apoptosis

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DISRUPTION OF CYTOKERATIN 18-CONTAINING INTERMEDIATE FILAMENTS IN BOVINE LUTEAL CELLS: EFFECTS ON FAS EXPRESSION, PROGESTERONE SECRETION, AND FASL-INDUCED APOPTOSIS

By

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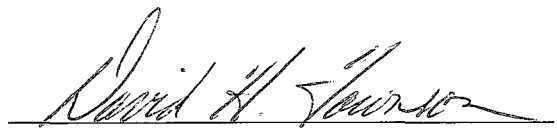

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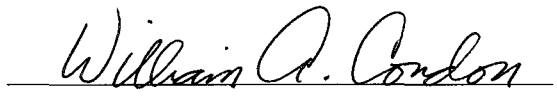

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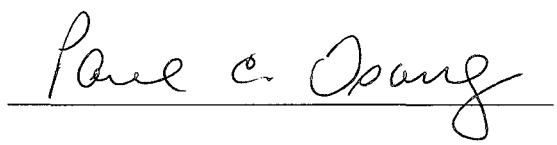

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ABSTRACT

DISRUPTION OF CYTOKERATIN 18-CONTAINING INTERMEDIATE FILAMENTS IN BOVINE LUTEAL CELLS: EFFECTS ON FAS EXPRESSION, PROGESTERONE SECRETION, AND FAS LIGAND-INDUCED APOPTOSIS

by

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In the current study, the possibility that cytokeratin 18 (CK18)–containing intermediate filaments (IFs) protect bovine luteal cells from FasL-induced apoptosis was explored. Bovine corpora lutea (CL) from early and late stages of the luteal phase were collected and prepared for cell culture. The cultures were exposed to culture medium without (control) or with 5mM acrylamide for 4 hr to disrupt CK18 IFs. Subsequently, the cultures were exposed to fresh medium without (control) or with FasL (50ng/mL) for 24 hr to induce apoptosis. Acrylamide disrupted CK18 IFs without affecting microtubules, progesterone secretion ($P>0.05$), or cell viability ($P>0.05$). Surface expression of Fas receptor was relatively high on luteal cells from early and late stage CL (84% and 61% of cells, respectively). Disruption of CK18 IFs did not enhance this ($P>0.05$), nor did it augment FasL-induced cell death ($P>0.05$). The results indicate CK18 IFs do not protect bovine luteal cells from FasL-induced apoptosis.

CHAPTER I

LITERATURE REVIEW

Consequences of Improved Lactation Efficiency on Dairy Cattle Fertility

The global demand for dairy products such as cheese, butter, and milk has increased over the past decade. Increased fluid milk production has largely met this demand, although at the expense of cow fertility (1-4). In the United States dairy industry, selective breeding and genetic improvement have provided for a steady increase in milk production over the years. More specifically, milk production has nearly doubled since 1960, increasing from 6252 to over 11,000 kg/year/cow (2). This increase in milk production per cow, although beneficial to the dairy industry, has come at a cost: milk production has increased while reproductive performance has declined. The decline in reproductive performance includes an increase in the number of services per conception, or the number of times a cow is inseminated before successful fertilization, and an increase in the number of days open, or the number of days from calving to subsequent fertilization (2). Although a link between high milk production and decreased fertility has been suggested, there are other likely factors contributing to the decline in reproductive performance, such as management, disease, and nutrition (1). Nevertheless, a substantial number of dairy cows encounter reproductive failure, as noted by a survey conducted by the United States Department of Agriculture. In this survey, 26.3% of US dairy cattle were culled due to reproductive problems, such as failure to ovulate or

conceive (3). Resolving this issue of declining reproductive performance in cows requires a thorough understanding of the physiological mechanisms that mediate ovulation and maintenance of pregnancy.

Bovine Estrous Cycle

The bovine estrous cycle lasts approximately 21 days and is generally divided into three stages: the follicular phase, the estrous phase, and the luteal phase. Within the ovary are structures called follicles, which are responsible for nurturing individual eggs, or oocytes. Follicles consist of a granulosal cell layer, a basement membrane, and a thecal cell layer. During the follicular phase of the estrous cycle, follicles are stimulated to mature in response to luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH stimulates androgen synthesis within thecal cells, while FSH acts on granulosal cells by stimulating the conversion of androgens into estradiol (5). The increase in estradiol stimulates estrous behavior (estrous phase) and a surge in LH, which signals the rupture of the mature follicle. The rupturing of the follicle leads to the release of the oocyte, a process called ovulation (5-7). After ovulation, the collapsed follicle undergoes a transformation to become a progesterone-secreting structure called the corpus luteum (CL) (6-10). In cattle, gestation lasts between 280 and 285 days and the corpus luteum is responsible for maintaining pregnancy throughout most of this period (5). If pregnancy does not occur, the corpus luteum regresses, stimulating the start of another estrous cycle. However, the untimely loss of CL function has been implicated in spontaneous embryonic loss and the decline in fertility of the dairy cow.

Corpus Luteum Formation, Function and Regression

Formation and Maintenance

The transformation of the follicle into the CL is a process called luteinization, in which granulosal and thecal cells transform into luteal cells that constitute the CL. It is generally believed that granulosal cells transform into what are known as large luteal cells, and thecal cells transform into small luteal cells (11). The suggestion that small luteal cells transform into large luteal cells as the CL matures has also been put forth (11,12). The primary function of the CL is to secrete the hormone progesterone, which is produced in response to the anterior pituitary-derived hormone LH (9,13). In vitro, large luteal cells produce higher amounts of progesterone than small luteal cells (14); however, large luteal cells are also relatively un-responsive to LH. In contrast, small luteal cells are responsive to LH, and thus produce comparable amounts of progesterone to large luteal cells when exposed to LH (14,15). The lifespan of the CL, called the luteal phase, can be divided further into four distinct stages based on progesterone production and appearance of the CL. Stage I (early stage) consists of newly formed CL in which the tissue secretes relatively low concentrations of progesterone (i.e., 1.5ng/mL) and is relatively small. Stages II and III (mid stage) CL produce the maximum amount of progesterone (i.e., 6.9-7.8ng/mL) and are larger in size (8,16). The secretion of high amounts of progesterone by stage II and III CL not only promotes embryonic implantation following conception, but is also thought to prevent the death of luteal cells (17). Finally, in the absence of pregnancy, stage IV (late stage) CL are characterized by a decline of progesterone production (i.e. 1.2ng/mL) and a decrease in tissue size, which marks the start of regression of the CL, an event called luteolysis (8,16).

Events During the Regression of the Corpus Luteum

Luteolysis is defined as the regression of the CL during which a decline in progesterone production and a decline in luteal mass occurs. In cattle, the pulsatile release of uterine-derived prostaglandin F2α (PGF2α) is believed to trigger the initial decline of progesterone (10,13,18). PGF2α is known to decrease blood flow to the CL (10), decrease plasma membrane fluidity of luteal cells (13,19,20), and disrupt lipoprotein-stimulated steroidogenesis (progesterone production) (21,22). The decline in size of the CL is triggered by a mechanism of cell death called apoptosis. During the regression of the CL, luteal cells (23) and endothelial cells (24) undergo apoptosis, contributing to the demise of the corpus luteum.

Mechanisms of Apoptosis

Programmed cell death, or apoptosis, is central to homeostasis of tissues. The term apoptosis was coined by Kerr, Wyllie, and Currie in 1972, who suggested that apoptosis plays an important role in tissue kinetics (25). Apoptosis is characterized by changes in mitochondrial function (26), increases in phosphatidyl serine on the outer leaflet of the plasma membrane (26,27), nuclear and DNA fragmentation (25-27), increases in plasma membrane permeability (26), membrane blebbing (25-27), and cytokeratin-intermediate filament cleavage (28-32).

As is evident from the above description, the mechanisms of apoptosis are complex, but generally there are two primary pathways involved: the extrinsic, death receptor pathway and the intrinsic, mitochondrial pathway. In the extrinsic pathway,

ligation of a death receptor leads to the recruitment of a death domain, which activates a series of caspases, and ultimately leads to the induction of apoptosis (27,33). In the intrinsic pathway, stimuli such as radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals lead to the opening of the mitochondrial permeability transition (MPT) pore. The opening of the MPT pore eventually results in the activation of caspases, inducing apoptosis (27,33). Within a regressing CL, the intrinsic and extrinsic pathways work in concert to induce luteal cell death after PGF2α treatment (34). Luteal cells exhibit tell-tale signs of apoptosis, with increased frequency of oligonucleosomes (fragmented DNA) and fragmented nuclei (8,23,35,36).

The process of apoptosis induces striking changes in cellular morphology, and within the bovine CL, the regulation of programmed cell death is considered dependent on immune system components. This complex interaction between the immune system and CL has been the topic of many studies, from which it has been found that signaling molecules (cytokines) contribute to morphological changes during CL regression. As the CL undergoes luteolysis, the cytokines tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and Fas ligand (FasL) contribute to decreased cellular viability by inducing apoptosis. Collectively, these mechanisms are thought to decrease CL weight and impair progesterone production, the hallmarks of luteolysis (27).

Immune System Involvement During Regression of the CL

Immune Cells within the Corpus Luteum

The presence of immune cells within the bovine corpus luteum was first described in 1968 by Lobel and Levy (37). Since then, studies evaluating the expression of

immune cells during the regression of the CL have led to the conclusion that immune cells and their secreted peptides called cytokines contribute to luteolysis (38-41). Immune cell populations within the CL have been described in the horse (42), human (43), mouse (44), rabbit (45), dog (46) and cow (47-49). In horse, human, mouse, rabbit, and dog CL, an increase in T lymphocytes is evident before or at the time of luteolysis, which is accompanied by an increase in macrophages (42-46). Similarly, in bovine CL, T lymphocytes and macrophages increase before the onset of luteolysis (47-49). This influx of immune cells prior to luteolysis suggests that immune cells contribute to the regression of the CL. What is not entirely clear at present, however, is the mechanism(s) by which these cells become activated within the CL and the extent to which they influence regression.

Activation of Immune Cells

As immune cells invade the regressing CL, major histocompatibility complex (MHC) molecules are expressed on luteal cells and are thought to activate immune cells through direct cell-cell signaling. MHC molecules are glycoproteins found on the surface of target cells that bind T cells via a T cell receptor, rendering it activated (39-41,50). All nucleated cells express class I MHC molecules, which are recognized by CD8+ cytotoxic T cells. The expression of class II MHC molecules is usually limited to cells of the immune system and is recognized by CD4+ helper T cells (39-41,50).

Class I MHC molecules are found on the surface of luteal cells, possibly responsible for the activation of immune cells, and the expression of class I MHC molecules coincides with the recruitment of immune cells to the CL (49). More

specifically, class I MHC expression increases before the onset of luteolysis, and this coincides with an increase in the accumulation of monocytes, macrophages, and T lymphocytes (49). Additionally, class II MHC molecules are expressed in human, equine, and bovine CL, with elevated expression occurring at the time of luteolysis (42,43,47,51,52). Collectively, the presence of class I and II MHC molecules in bovine CL, with elevated expression during luteal regression, suggests these molecules activate immune cells and possibly trigger their recruitment during luteolysis.

Cytokine Secretion

Once activated, immune cells secrete cytokines, which can have cytotoxic effects on luteal cells, contributing to the regression of the CL. Studies evaluating the expression of mRNA encoding the cytokines TNFα and IFNγ reveal these two cytokines are present within the bovine CL throughout the estrous cycle (53,54). In late stage CL, however, reports of mRNA expression for TNFα are less consistent. One report (53) indicated TNFα mRNA did not change from mid- to late-stage CL. In another report, TNFα expression increased in regressed CL (54). Petroff et al. used d18 CL to characterize mRNA expression, whereas Korzekwa et al. utilized d19-20 CL, which may account for the discrepancy observed. During induced luteolysis, IFNγ mRNA expression increases immediately following PGF2α treatment (53,55). Initially, TNFα mRNA was reported to remain the same following induced luteolysis (53). More recently, however, TNFα mRNA expression was shown to increase following PGF2α treatment (55). One possible explanation for the varying reports could be a difference in materials and techniques utilized. The approached used by Neuvians et al. (2004) to quantify mRNA expression

has been shown to be highly accurate and much more sensitive than the approach used by Petroff et al (1999). It is proposed that these cytokines (TNFα and IFNγ) exert their effects on the CL by increasing prostaglandin synthesis and blocking gonadotropin-stimulated steroidogenesis, while initiating the mechanism of apoptosis (39-41). The presence of cytokine mRNA coupled with the infiltration of leukocytes and presence of class I and II MHC molecules in luteal tissue at the time of luteolysis, supports the concept that immune cells play a critical role in the regression of the CL.

Tumor Necrosis Factor-α

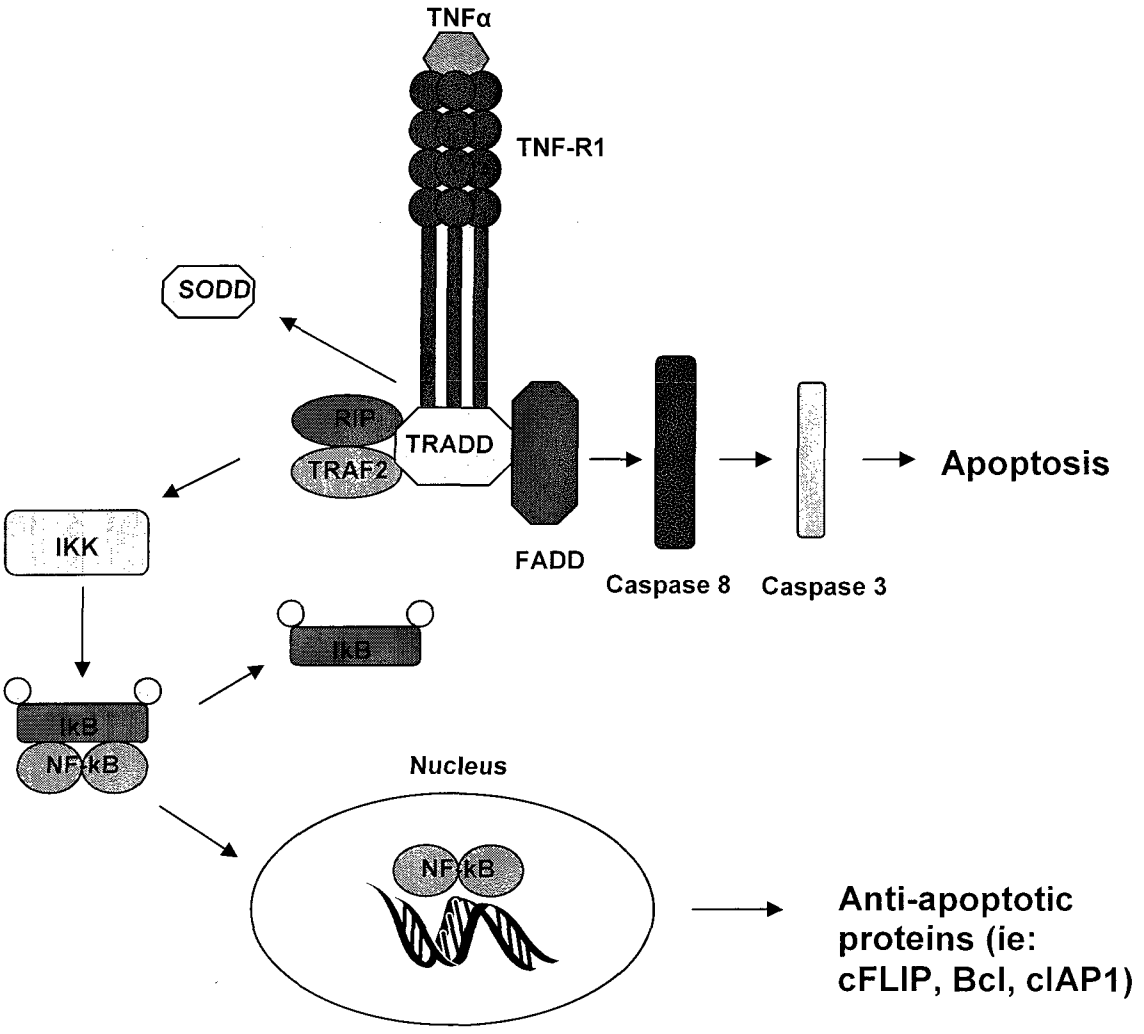
The cytokine Tumor Necrosis Factor-α (TNFα) was first described as an endotoxin-induced, secreted serum factor resulting in the necrosis of tumors (56). Following the discovery of TNFα, Mannel et al. determined the cellular source of TNFα to be macrophages (57). Although macrophages are viewed as the primary source of TNFα, other cell types within the ovary have been implicated as sources of TNFα secretion, such as endothelial cells (58), luteal cells (59), and granulosal cells (59,60).

TNFα exerts its effects on target cells by binding to two possible TNF receptors (TNF-R), TNF-R1 and TNF-R2. TNF-R1 is expressed in most tissues and activates gene expression or induces apoptosis. TNF-R2 is highly regulated and found on cells of the immune system where it promotes cell survival. Unlike TNF-R1, TNF-R2 is incapable of transmitting an apoptotic signal since it lacks an intracellular death domain. Interestingly, TNF-R2 is only fully activated by membrane-bound TNFα, whereas TNF-R1 is activated by membrane and soluble forms of TNFα, suggesting that TNF-R1 is the key mediator of TNFα signaling (61).

TNFα activates gene expression through TNF-R1 by triggering the nuclear translocation of the transcription factor nuclear factor kappa B (NF-κB). When TNFα binds to TNF-R1, SODD (Silencer of Death Domain) dissociates from TNF-R1, recruiting the adapter protein TRADD (TNF Receptor Associated Death Domain). TRADD allows for the binding of TNF-receptor associated factor 2 (TRAF2) and the kinase RIP (Receptor Interacting Protein). The I-κB kinase (IKK) complex is recruited to TRAF2, where it is activated by RIP, subsequently leading to the phosphorylation of I-κB, the inhibitory counterpart of NF-κB. The phosphorylation of I-κB releases NF-κB and allows for its translocation into the nucleus. NF-κB targets anti-apoptotic genes, such as cFLIP, Bcl, and cIAP1 (61) (see Figure 1).

Although TNFα stimulates gene expression, it also induces cell death. Similar to the pathway described above, TNFα binds to TNF-R1, resulting in the dissociation of SODD and the recruitment of TRADD. TRADD then binds to FADD (Fas Associated Death Domain), which triggers a series of caspase enzyme activated cascades. Pro-caspase-8 is recruited to FADD, where it is cleaved into its active form, caspase-8. Caspase-8 activates other pro-caspases, especially pro-caspase-3, which eventually leads to apoptosis (61) (See figure 1).

Figure 1. TNF Signaling Pathway



Within the corpus luteum, TNFα was originally described for its luteolytic actions, but more recently, a luteotropic role of TNFα has been revealed (62). The actions of TNFα on the secretion of prostaglandins appear to be dependent on the concentration of TNFα used in experiments. Treatment of cattle with high doses of TNFα (i.e. 10μg) stimulates progesterone production, lengthening the estrous cycle, and stimulates production of luteotropic prostaglandin E_2 (PGE_2). On the other hand, low doses of TNFα (i.e. 1μg) stimulates PGF2α secretion and the shortening of the estrous

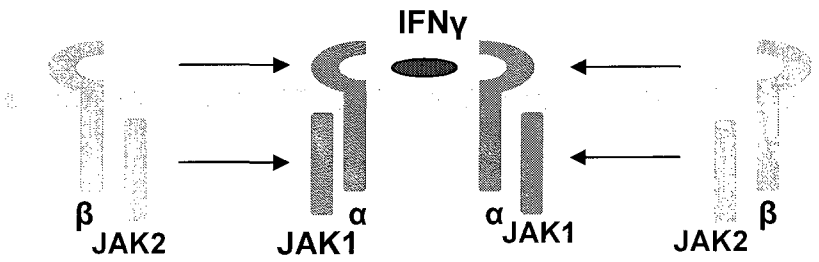
cycle (54,63-65). Although not completely understood, one plausible explanation for the control of the dual role of TNFα is the different TNF receptors. It is possible that the concentrations of TNFα dictate which receptor it binds to, with lower concentrations of TNFα preferentially binding to TNF-R1, inducing apoptosis, and higher concentrations of TNFα binding to TNR-R2, promoting survival (64).

Although TNFα has luteotropic actions, it is predominantly considered to have a role in the regression of the corpus luteum. As stated above, during the onset of luteolysis, immune cells infiltrate the CL, including macrophages. Samples collected from bovine CL using continuous-flow microdialysis reveal that TNFα protein concentration increases during the onset of luteolysis, but not until after the initial decline in progesterone (66). Similarly, Sakumoto et al. reported an increase in TNFα concentrations between days 13 and 18 of the estrous cycle (63). In studies evaluating mRNA, some found that TNFα mRNA is elevated in regressed and PGF2α treated CL (54,55), whereas others found no difference in TNFα mRNA throughout the estrous cycle (47,53,63). Potential methodological differences aside, the presence of TNFα mRNA and increased protein concentrations within the regressing bovine CL suggests a functional role for this cytokine during luteolysis. In vitro, TNFα inhibits LH-stimulated progesterone production and increases prostaglandin (PGF2α) synthesis in cultured bovine luteal cells (54,63,67). In porcine luteal cells, TNFα induces apoptosis (68), an effect seen only in bovine luteal cells when TNFα is administered concomitantly with IFNγ (67,69).

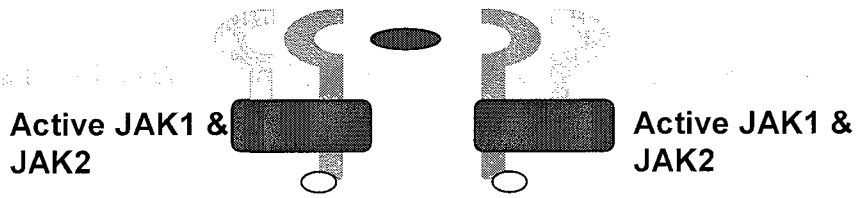
Interferon-γ

In 1957, Isaacs and Lindenmann described a substance responsible for protecting cells from viral infection, naming it the interferon (70). The interferon family is divided into type I and type II IFN. Type I IFN consists of IFNα and IFNβ, which are released as a result of viral infection. Type II IFN contains only one member, IFNγ, which is released in response to immune and inflammatory stimuli (71). IFNγ is a cytokine released by T lymphocytes and natural killer cells that induces its effects through Janus kinases (JAKs) and signal transducers and activators of transcription (STATS), forming a signal transduction pathway known as the JAK-STAT pathway. Once secreted, IFNγ binds to the IFN receptor (IFN-R), which contains a receptor α and β chain (IFN-Rβ and IFN-Rα, respectively). The IFN-Rα and IFN-Rβ chains are associated with inactive forms of the Janus family kinases JAK 1 and JAK2. After IFN-R ligation, the receptor dimerizes, resulting in the interaction between the IFN-Rα and IFN-Rβ chains and inactive JAK 1 and JAK2. The close association of JAK 1 and JAK2 provokes transactivation of one another. Active JAK1 and JAK2 enzymes phosphorylate the IFN-Rα chains of the receptor complex, creating "docking sites" for signal transducers and activators of transcription (STAT 1) molecules. The docking sites on the IFN-Rα chains attract two STAT 1 molecules, which are subsequently phosphorylated by the IFN-Rα chains. The STAT 1 molecules dissociate from the receptor complex, creating a homodimeric complex which then translocates into the nucleus, activating gene transcription (71) (See figure 2).

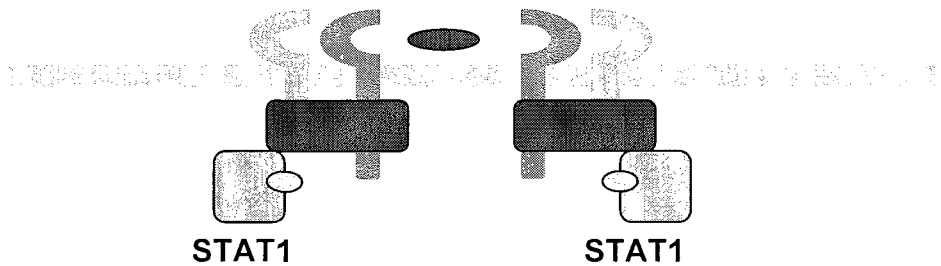
Figure 2. IFN Signaling Pathway



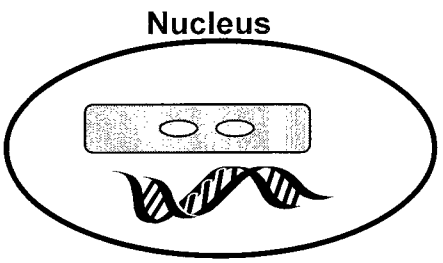
Step1: As IFNγ binding occurs, the receptor dimerizes (arrows), bringing together the IFN-Rα and IFN-Rβ chains, as well as inactive JAK1 and JAK2.



Step 2: The close association of JAK1 and JAK2 leads to the transactivation of one another (red). Active JAK1 and JAK2 enzymes phosphorylate the IFN-Rα chains.



Step 3 and 4: The phosphorylated sites on the IFN-Rα chains recruits STAT1 molecules, where they are activated. Following activation, the STAT1 molecules dimerize and translocate into the nucleus.



IFNγ is synthesized in response to immune and inflammatory stimuli and is thought to play an important role in the regression of the CL. Throughout the estrous

cycle and after PGF2α treatment, IFNγ mRNA is present in the bovine CL (47,53,55). In murine ovaries, IFNγ detection is strongest during structural regression of the CL, which corresponds with a peak in T lymphocyte infiltration (44). In cultured bovine luteal cells, treatment with IFNγ significantly increases the expression of class I and class II MHC molecules (51) while inducing PGF2α secretion (72). Additionally, IFNγ inhibits LH-stimulated steroidogenesis, but has no effect on basal progesterone secretion (72). IFNγ alone induces cell death (69,72), but the incidence of cell death is enhanced by treatment with IFNγ and TNFα concomitantly (67,69). The synergistic effects of IFNγ and TNFα in vitro suggest these two cytokines may act in concert to enhance regression of the CL in vivo.

Fas-FasL

The discovery of the Fas/Apo-1/CD-95 antigen occurred in 1989 when two separate groups identified a monoclonal antibody with death-inducing capabilities (73,74). After isolating the cDNA encoding the Fas antigen, it was determined that the Fas antigen was similar to TNF-R1, TNF-R2, and CD40, suggesting that Fas is a member of the TNF receptor superfamily (75). The Fas receptor and its ligand, FasL, is a system originally described for its function in the immune system (73,76-79), and areas of immune privilege, such as the testes (80) and eye (81). More recently, the Fas-FasL system is recognized for a vital role in tissue homeostasis throughout the body.

The Fas receptor (Fas-R) shares a homologous cytoplasmic death domain with TNF-R1 that transmits a death-inducing signal (82). Fas-R is expressed on the surface of cells, however, reports indicate that Fas-R resides intracellularly until signaled to

mobilize to the surface of the cell (83-85). When expressed on the surface of cells, FasL binds to Fas-R, inducing an apoptotic signal. Two cell types have been identified based on their intracellular transmission of the apoptotic signal induced by FasL: type I and type II cells (86).

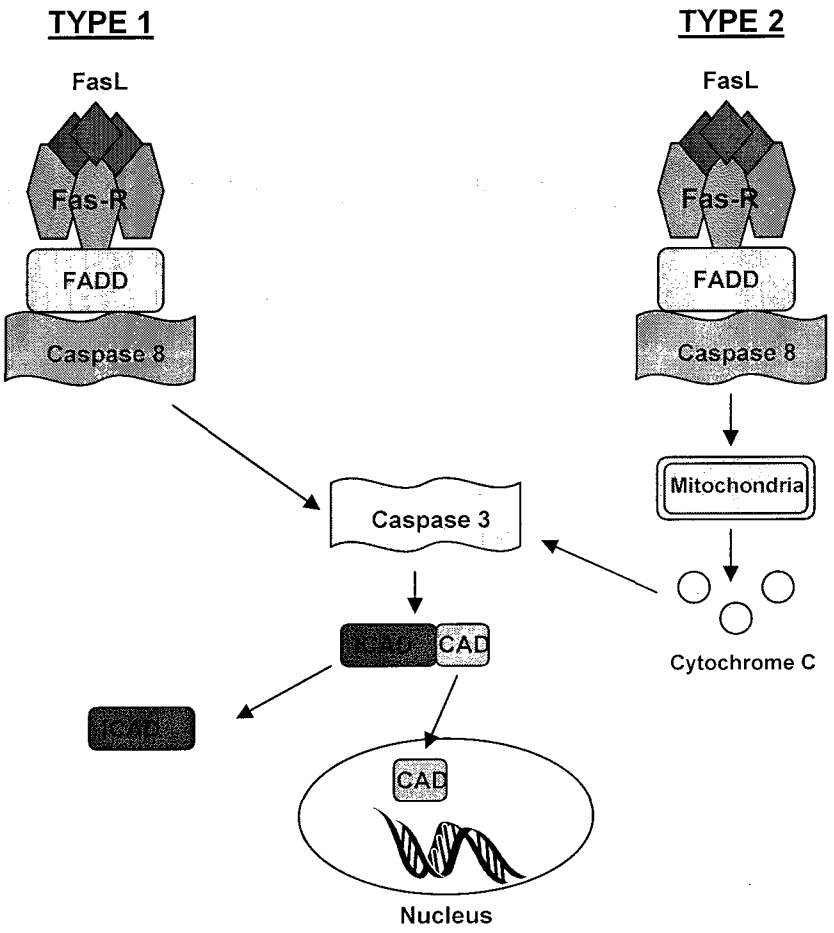
In type I cells, binding of FasL leads to activation of the caspase cascade, and induction of apoptosis. In order to transduce the apoptotic signal, Fas-R must trimerize after stimulation with FasL. The clustering of Fas-R recruits the adaptor protein FADD. FADD contains a death domain at its C terminus, which interacts with Fas-R. The death effector domain (DED), located at the N terminus of FADD, binds procaspase-8. Fas-R, FADD, and caspase-8 form a protein complex known as the death inducing signaling complex (DISC). The self-cleavage of procaspase-8 signals the caspase cascade, which leads to the activation of procaspase-3 and apoptosis (82,86) (See figure 3).

In type II cells, ligation of Fas-R induces apoptosis, but the intracellular mechanisms are different from those of type I cells. Clustering of Fas-R occurs after stimulation with FasL, which recruits FADD and procaspase-8 (the DISC). However, in type II cells, the formation of the DISC is reduced as compared to type I cells, suggesting that there is a decreased amount of activated caspase-8. Instead of caspase-8 primarily activating procaspase-3, it is responsible for changes observed in the mitochondria. There is a loss of mitochondrial transmembrane potential, formation of permeability transition pores, and release of cytochrome c into the cytoplasm. The influx of cytochrome c into the cytoplasm leads to the activation of procaspase-3 and subsequent cell death. In type II cells (e.g. CEM T-cell line), the requirement of the mitochondria to induce cell death was demonstrated by over-expressing the mitochondrial suppressor,

Bcl-2 (86). In cells with over-expression of Bcl-2, there is a decrease in procaspase-8 and procaspase-3 activation. This is accompanied by a decreased sensitivity to FasL. Additionally, over-expression of Bcl-2 in type I cells does not affect caspase activation or the incidence of cell death, giving strong support to the concept that the mitochondria is essential in transmitting the death signal in type II cells (86) (See figure 3).

In both type I and type II cells, FasL induces cell death through a series of signaling pathways within the cells. One of the defining characteristics of apoptosis is the degradation of chromosomal DNA. Activation of Fas-R leads to the cleavage of DNA through caspase-activated DNase (CAD). In unstimulated cells, CAD is complexed with its inhibitor, ICAD. However, once FasL stimulates Fas-R, procaspase-3 is activated, resulting in the cleavage of ICAD and the release of CAD into the nucleus, where it cleaves DNA (82,87). Additionally, as described above, caspase-3 is responsible for the cleavage of the cytoskeletal element cytokeratin 18 (28-32). The intracellular signaling pathway triggered by FasL stimulates the enzymatic cleavage of several products, leading to the tell-tale signs of apoptosis (See figure 3).

Figure 3: Fas Signaling Pathway



Although originally described for its role in the immune system, the Fas-FasL system has been described in several other tissue types, including the liver (hepatocytes) (84,85,87), lung epithelial cells (88) and the ovary (69,89-102). Within the ovary, researchers have investigated the role of the Fas-FasL system in folliculogenesis and corpus luteum function. Follicles, granulosal cells and thecal cells express the Fas antigen (91,96,98). Furthermore, FasL and Fas mRNA are increased in granulosal cells of early atretic follicles as compared to healthy follicles. Additionally, FasL and Fas protein expression also increases in granulosal cells of progressed atretic follicles as

compared to healthy and early atretic follicles (99). In the bovine CL, Fas mRNA increases in the regressed CL (69), and both Fas and FasL mRNA increase within two hours of PGF2α treatment (102). In rat CL of pregnancy and postpartum, Fas and FasL protein as well as FasL mRNA increase on day 22 of pregnancy and remain elevated through day 1 postpartum, the time at which the CL would start to naturally regress (93). Finally, in rat CL treated with the luteolytic agent prolactin, FasL protein expression in the membrane-bound and soluble form is increased (92). The presence of Fas and FasL in ovarian structures suggests that this system influences follicular atresia and luteolysis.

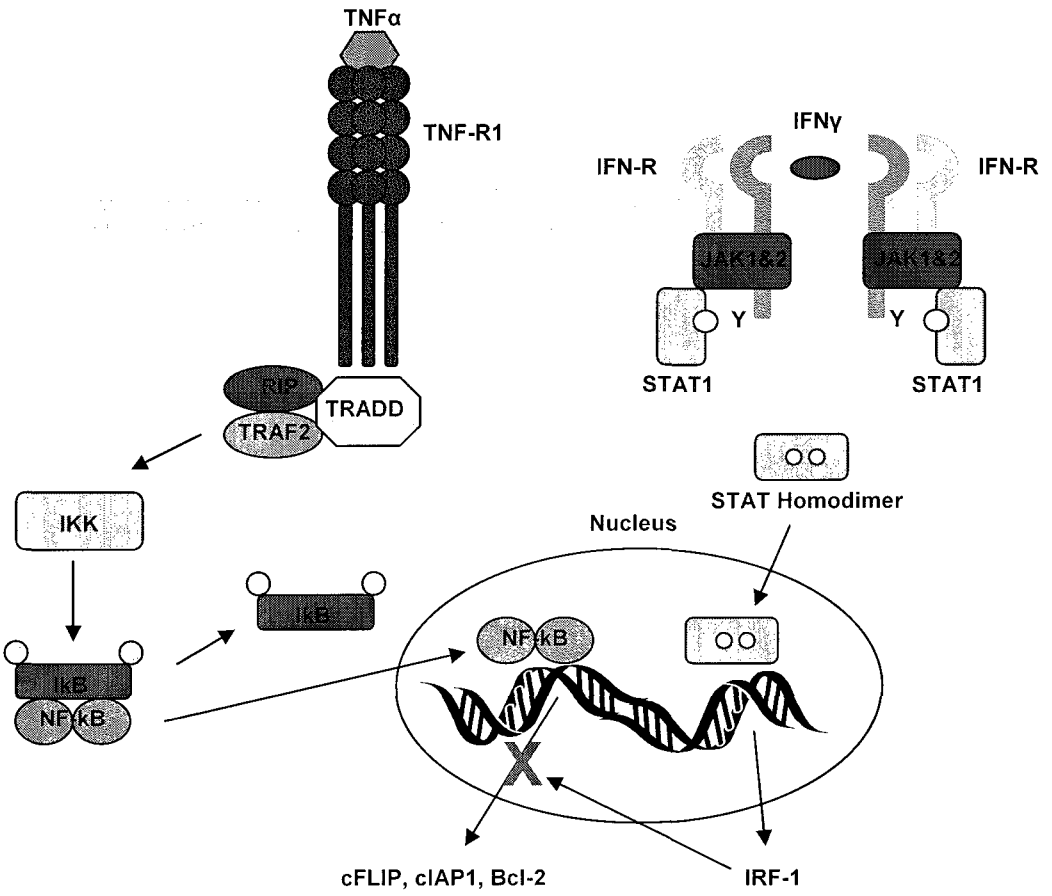
The source of FasL within the ovary appears to be the immune cells that infiltrate at the time of luteolysis. In rat CL, luteal cells can be separated into two populations based on size and density. Immune cells, which lack steroidogenic activity, clearly express FasL mRNA (94). In contrast, steroidogenic cells lack FasL, but express high amounts of Fas mRNA (94). The specific source of FasL is believed to be T cells, as demonstrated by the increase of membrane-bound and soluble FasL protein as a result of treatment with the T cell activator concanavalin A (ConA) (94). Although Fas and FasL are present in CL of different species at the time of luteolysis, FasL is not cytotoxic unless combined with IFNγ and TNFα, suggesting a synergistic role for these three cytokines (69,89,90,95).

TNF, IFN, FasL

As mentioned above, TNFα and IFNγ synergistically induce cell death in several cell types (67,69,103-105). One possible explanation for this effect is the observation that IFNγ induces TNF-R1 and TNF-R2 expression on the surface of epithelial and

myeloid cell lines (106). This increase in TNF-R expression occurs in a dose-dependent manner, and is not accompanied by a change in receptor affinity (106). Additionally, the activation of STAT1 by IFNγ is a critical step for the synergistic effects of TNFα and IFNγ (107). Activation of STAT1 stimulates the production of interferon regulatory factor 1 (IRF1), which then inhibits NF-κB activity. Collectively, these signals block transcription of anti-apoptotic genes. The inhibition of NF-kB primes cells for TNFα-induced effects, resulting in increased cell death (108) (See figure 4). In luteal cells, another possible explanation for the synergy of IFNγ and TNFα is the ability of these cytokines to stimulate PGF2α secretion in culture (105). Increases in PGF2α may be a contributing mechanism to the decreased viability of luteal cells co-cultured with IFNγ and TNFα.

Figure 4: TNF and IFN Signaling Pathway



IFNγ and TNFα decrease cell viability, but these cytokines also act in concert with Fas-R and the Fas-FasL system. In cells treated with IFNγ and TNFα, Fas mRNA (69,96,101,109,110) and protein expression increases (74,104,110). In addition to augmenting Fas mRNA and protein expression, IFNγ and TNFα increase cell susceptibility to FasL-induced death. Ovarian cells are more prone to death when treated with IFNγ, TNFα, and FasL as compared to FasL alone, suggesting that these cytokines mediate FasL-induced cell death (69,96,109).

There are several complex mechanisms controlling the regression of the CL. As described above, multiple systems work in concert to decrease progesterone production and induce cell death. During regression of the CL, the induction of apoptosis is a very

cell specific process (111). Immunohistochemical staining for fragmented DNA shows that individual cells will undergo apoptosis while neighboring cells appear to be unaffected (111). To further understand the cell-specific mechanisms controlling luteal cell death, some are investigating the role of intermediate filaments as a possible mediator of apoptosis within the CL.

Cytoskeleton and Intermediate Filaments

The cytoskeleton of cells consists of microtubules, which are the largest diameter filaments; microfilaments, which are the smallest diameter filaments; and finally, intermediate filaments with a diameter that ranges between 7 and 11 nm (112). Intermediate filaments consist of a family of five different subtypes, which includes cytokeratins, vimentin, desmin, neurofilaments, and glial filaments. Cytokeratin-like proteins (CK), are generally found in epithelial cells. Vimentin filaments are found in a variety of cell types, including sertoli cells, vascular smooth muscle cells, and a variety of cell lines. Desmin filaments are found in myogenic cells, while neurofilaments comprise neuronal cells, and glial filaments provide structure for astrocytes (112). Vimentin, desmin, and glial intermediate filaments are composed of one type of subunit protein, whereas cytokeratin filaments are obligate heterodimers. The dimers forming cytokeratin filaments consist of an acidic cytokeratin (type I, K9-K20), and a basic cytokeratin (type II, K1-K8) (112,113). The combination of the type I and type II cytokeratins determines the nomenclature for that particular intermediate filament. For example, an intermediate filament consisting of K8 type II cytokeratin and K18 type I cytokeratin is named cytokeratin 8/18, or CK-8/18. Tissue and cells express cytokeratin intermediate filaments

composed of different combinations of these dimers (i.e. CK-8/18, CK-8/19 etc.), which serves as an "identification tag" for that particular tissue or cell type (114).

Throughout the years, several different potential functions of intermediate filaments have been described. Intermediate filaments provide mechanical integrity to cells, contribute to cell stiffness, stiffening behavior, and proliferation. They also act as an anchor to desmosomes (intercellular junctions) at the cell membrane, aid in the movement of lipid droplets in steroidogenic cells, and play an important role in cell spreading (113,115-118). To investigate the role intermediate filaments play in cell stiffness and stiffening behavior, coated beads and a magnetic cell twisting device have been utilized to induce stress and strain on cultured cells. Cells with vimentin filaments are more resistant to twisting and have a greater stiffness than vimentin-deficient cells (118). However, treating with acrylamide, an agent known to disrupt intermediate filaments, causes vimentin-positive cells to gain characteristics similar to vimentin-deficient cells in regards to stiffening behavior in response to stress (118). This indicates intermediate filaments influence cellular response to stress. Additionally, vimentin-deficient cells have slower rates of proliferation than wild type cells, as evidenced by reduced rates of DNA synthesis (118).

Intermediate filaments also interact with several types of intracellular proteins. One example is the interaction of intermediate filaments with the plaque protein of desmosomes (115). Intermediate filaments provide anchorage for the desmosome, allowing for intercellular contact. This association is found between cells in stratified squamous and simple epithelia, epithelial cells in culture, and in carcinomas (115). In addition to desmosomes, there is a close association between vimentin intermediate

filaments and lipid droplets (117). Lipid droplets are an essential component of the steroidogenic pathway. Lipid droplets contain cholesterol ester, which is utilized by the mitochondria to synthesize pregnenolone, a precursor for the hormone progesterone. Due to the close association among vimentin, lipid droplets, and the mitochondria, it is thought that intermediate filaments influence the movement of cholesterol during steroidogenesis. Supporting this hypothesis, cells that lack vimentin are unable to utilize cholesterol to the same extent as vimentin containing cells (117).

Finally, intermediate filaments are implicated in the process of epithelial cell spreading. In rounded cells, a coiled cap of intermediate filaments is present (116). As the cell spreads, these intermediate filaments migrate from the cap and expand the cytoplasm. After the initiation of cell spreading, the cap is no longer apparent, but becomes visible again after the intermediate filaments have retracted closer to the nucleus. The formation and disappearance of the intermediate filament cap is suggested to be under the control of the intermediate filament organizing center, renamed the intermediate filament distribution center (IFDC) (116).

Role of Intermediate Filaments in Apoptosis

Intermediate filaments, specifically cytokeratin intermediate filaments in simple epithelia, are necessary for the development of mid-gestational mouse embryos. Deleting the keratin 8 gene in mouse embryonic stem cells prevents filament formation and causes lethality at 12-13 days of development (119). Intermediate filament expression determines the fate of embryos, but it also plays an important role in cell death (apoptosis) as well as survival.

During apoptosis, the cytokeratin 18 subunit of CK-8/18 filaments is the target of caspase cleavage (120,121). Cytokeratin 18 is cleaved by effector caspases (i.e., caspase-3) at Asp237 (VEVD) and Asp396 (DALD) (32,120,122) (See figure 5). The cleavage of cytokeratin 18 is a useful marker for patients suffering from degenerative liver diseases, such as acute liver failure, cirrhosis, and cancers, because as hepatocytes (liver cells) undergo apoptosis, cytokeratin 18 is cleaved and diffuses into the serum. Measuring the concentrations of cytokeratin 18 in the sera of these patients permits monitoring of the severity of the disease in a non-invasive way (30,31).

Figure 5: Caspase Cleavage of Cytokeratin 18 (Tao et al., 2008)

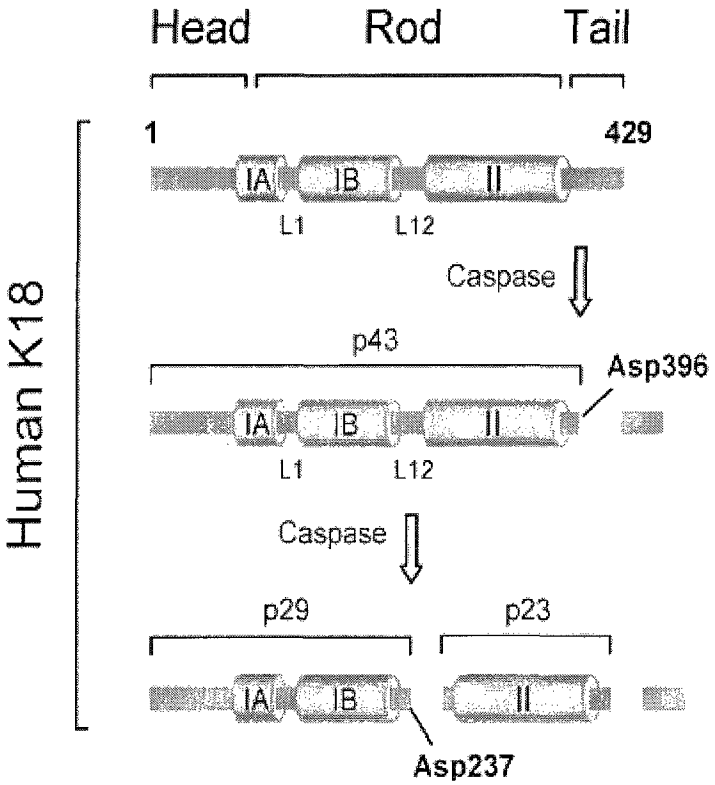


Diagram of human cytokeratin 18. Represented is the head, rod, and tail domain of K18. The rod domain consists of three sub-domains: IA, IB, and II, which are separated by the linker L1 and L12. p43, p29, and p23 represent sites of caspase cleavage (Tao et al., 2008).

When a cell undergoes apoptosis, the cleavage of cytokeratin 18 by caspase-3 leads to the collapse of the intermediate filament structure and subsequent formation of cytoplasmic inclusions. The colocalization of cytokeratin 18 and caspase 3 within these inclusions verifies their close association during the process of apoptosis (123). Recently, the mechanism of caspase-mediated cytokeratin intermediate filament disruption has been investigated, leading to the identification of a new protein that influences caspase-3 and CK-18 interactions.

Caspase-3 is recruited to cytokeratin intermediate filaments through a newly identified protein called death effector domain containing DNA binding protein (DEDD) (124). The molecule DEDD contains nuclear localization signals, but is found abundantly within the cytosol. Immunostaining of DEDD within cells reveals a filamentous pattern, resembling intermediate filaments (124). As apoptosis progresses, DEDD is no longer filamentous, but rather is expressed in a more aggregated fashion contained within cytoplasmic inclusions. While DEDD colocalizes with cytokeratin filaments, it is also associated with active caspase-3. This suggests that DEDD forms complexes with active caspase-3, therefore mediating the recruitment of procaspase-3 to cytokeratin filaments for digestion during apoptosis (122,124).

Although DEDD is responsible for the recruitment of caspase-3 to intermediate filaments, the eukaryotic translation initiation factor 3 (eIF3) subunit, eIF3k, is responsible for its release from inclusions. eIF3k is associated with cytokeratin intermediate filaments in healthy cells (125). In cells undergoing apoptosis, eIF3k colocalizes with cytokeratin 18 within the cytoplasmic inclusions. When eIF3k is blocked by silencing RNA (siRNA), cells are less susceptible to cell death, with a decrease in ICAD (inhibitor of caspase-activated DNase) cleavage, a step required for the fragmentation of DNA (125). Downregulation of eIF3k also leads to the sequestration of caspase-3 within the inclusions containing fragmented cytokeratin 18. Thus, it is suggested that eIF3k releases caspase-3 from the cytoplasmic inclusions, allowing caspase-3 to continue cleaving additional filaments (125). This complementary role of DEDD and eIF3k enables efficient degradation of cytokeratin intermediate filaments, leading to a loss in cell structure and function.

Role of Intermediate Filaments in Cell Survival

The degradation of cytokeratin intermediate filaments during programmed cell death is an essential process during apoptosis, but it has been suggested that cytokeratin intermediate filaments are also capable of protecting cells from apoptosis induced by TNFα and FasL. Other cytoskeletal components, such as microtubules and microfilaments, have been implicated in resistance to death (126), but intermediate filaments may be responsible for the sequestration of important intracellular proteins required for the progression of apoptosis.

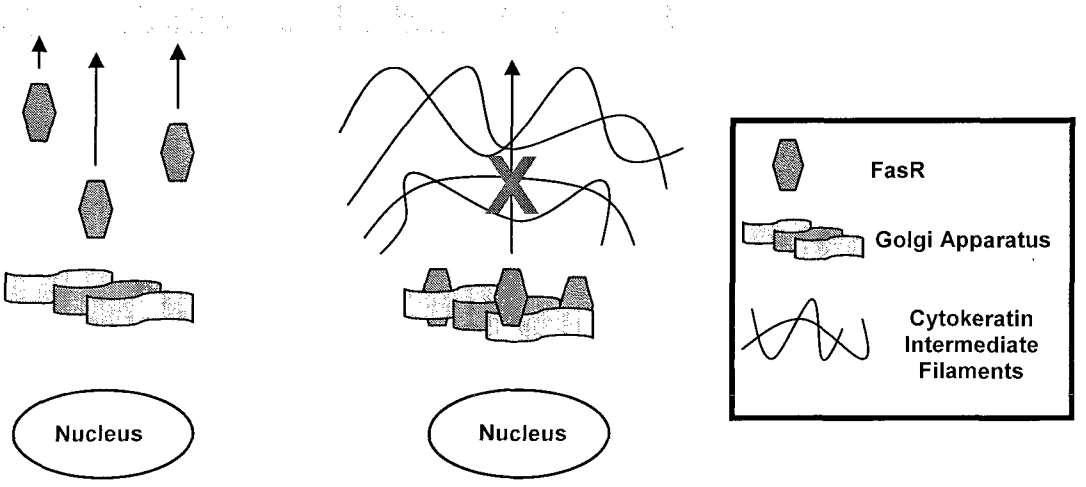
The influence of cytokeratin intermediate filaments on survival was first suggested after the observation that epithelial cells deficient in cytokeratin 8 or cytokeratin 18 are 100 times more sensitive to TNFα-induced death than cells expressing intact cytokeratin 8/18 intermediate filaments (127). Previously, it was reported that ligation of TNF-R2 induces cell death through stimulation of TNF-R1 (128) A close association between the NH_2-terminus of cytokeratin 18 and the cytoplasmic domain of TNF-R2 was found, providing a potential mechanism for the cytokeratin-dependent resistance to TNFα induced apoptosis (127).

To further investigate the role cytokeratin intermediate filaments play in resistance to cell death, Inada et al. (129), evaluated the interaction between TRADD (TNF receptor associated death domain) and cytokeratin 18. In unstimulated cells, TRADD and cytokeratin 18 are associated, but in response to high doses of TNFα, TRADD dissociates from cytokeratin 18 and interacts with TNF-R1. In the presence of TNFα, while TRADD is still associated with cytokeratin 18, viability is relatively high. As the dose of TNFα increases and TRADD interacts with TNF-R1, viability decreases.

These observations suggest that in addition to the association between cytokeratin 18 and TNF-R2, cytokeratin 18 is also capable of sequestering TRADD, thus protecting cells from TNFα-induced cell death (129).

In addition to TRADD, cytokeratin 18 is also responsible for the sequestration of Fas within the Golgi apparatus in murine hepatocytes. In cytokeratin-8 knockout hepatocytes, Fas is expressed mostly at the surface of the cells, whereas in cells expressing intact cytokeratin 8/18 filaments (wild type cells), Fas is localized mostly within the Golgi apparatus (85). Originally, it was described that the differential expression of Fas and cytokeratin in these two cell types (knockout vs. wild type) lead to a change in viability in response to treatment with Jo2, a Fas antagonist. Cells lacking cytokeratin intermediate filaments experienced higher levels of apoptosis than cells expressing intact filaments (85). After further investigation, it was discovered that in order to induce cell death in type II cells (hepatocytes), Jo2 has to be paired with protein A (PA). After this discovery, the relationship between cytokeratin intermediate filaments and Jo2+PA-induced cell death was re-evaluated. Contrary to previous findings, Jo2+PA did not induce higher levels of death in cytokeratin-8 knockout cells as compared to wild type cells; however, the kinetics of cell death differed between the two cell types. Cells lacking intermediate filament expression exhibit increased rates of apoptosis, DISC formation, and caspase-8 activity as compared to cells with intact intermediate filaments (130). The increase in mobilization of the Fas receptor and change in death kinetics as a result of intermediate filament loss leads to increased susceptibility to cell death in murine hepatocytes (see review: 131) (See figure 6).

Figure 6: Cytokeratin-mediated Fas Trafficking



Localization of Intermediate Filaments within the Ovary

The expression of intermediate filaments in varying cell types has been explored extensively, however, only a few investigations have described expression of intermediate filaments in ovarian tissues. Intermediate filament expression in the ovary has been characterized in human (132), rat (133), emu (134) and cow (135,136).

In fetal human ovaries, cytokeratin intermediate filament expression is found in the surface epithelium, sex cords, rete ovarii, and primordial and primary follicles. In adult human ovaries, cytokeratin is expressed in epithelial cells, and primordial and primary follicles, with no expression in preantral follicles. Additionally, nearly half of all luteal cells from early stage CL express cytokeratin (132). In rat ovaries, cytokeratin expression is limited to follicles, ovarian surface epithelium and CL, with no change in expression observed with age of the CL (133). Vimentin expression is also observed in rat ovaries, with expression found in thecal cells of follicles, ovarian surface epithelium, blood vessels, and CL (133). In the emu, vimentin expression is found in granulosal cells of developing and early atretic follicles, the ovarian surface epithelium, and endothelial

cells (134). In the cow, desmin, vimentin, and cytokeratin intermediate filaments are present in the ovary of fetuses, prepubescent heifers, and cows (136). Desmin is detectable primarily in smooth muscle cells surrounding blood vessels, but is not found in any other cell-type within the ovary (136). Vimentin is more evident than desmin, and occurs in epithelial cells of the cortical cords, fibroblasts, and primary, secondary, and antral follicles during fetal development, and in prepubescent heifers, and cows (136). Cytokeratin intermediate filament expression is restricted to the rete ovarii, ovarian surface epithelium, cortical cord epithelium, and primordial follicles before 7.5 months of gestation. In adult heifers and cows, cytokeratin is found in the rete ovarii, surface epithelium of follicles, and cortical cords (136).

In bovine CL, the expression of cytokeratin intermediate filaments varies depending on the age of the CL. Evaluation of more than 45 corpora lutea from adult cows revealed that cytokeratin expression is highest in early stage CL and lowest in the regressed, late stage CL. In early stage CL, there were approximately 61 ± 13 cells per field of view that stained positively for cytokeratin. Expression decreased to only 1 ± 1 cells per field of view positive for cytokeratin in regressed stage CL. Immunoblotting confirmed the immunohistochemical results, showing decreased protein expression as the CL ages (135, 137).

CHAPTER II

DISRUPTION OF CYTOKERATIN 18-CONTAINING INTERMEDIATE FILAMENTS IN BOVINE LUTEAL CELLS: EFFECTS ON FAS EXPRESSION, PROGESTERONE SECRETION, AND FAS LIGAND-INDUCED APOPTOSIS

Introduction

Since 1960, fertility in dairy cows has been declining, as indicated by an increase in the number of services per conception and an increase in the number of days open (2). Inadequate dairy management, increased frequency of disease, and increased metabolic disorders, many of which are a consequence of high milk production, are all contributing factors to the decline in reproductive performance (1,2). Resolving the issue of declining fertility in the bovine requires a greater understanding of the physiological mechanisms that mediate ovulation and the maintenance of pregnancy.

The corpus luteum (CL) is a transient ovarian structure in the cow responsible for maintaining pregnancy. The lifespan of the CL, called the luteal phase, is generally divided into four distinct stages based upon appearance of the CL and the amount of progesterone the CL secretes systemically (16). Early stage CL (stage I) are relatively small in size and secrete low concentrations of progesterone. Mid-stage CL (stage II and III) are larger in size and produce the maximum amount of progesterone. The high amount of progesterone secreted by the CL at stages II and III promotes implantation and the maintenance of pregnancy if conception occurs. If pregnancy does not ensue, the CL advances to a stage of regression (luteolysis), in which the size of the CL decreases precipitously and progesterone production declines (late stage; stage IV). However, an

untimely loss of CL function has also been implicated in spontaneous embryonic loss and an increase in infertility in the dairy cow.

In cows, the mechanism responsible for regression of the CL includes the pulsatile release of uterine-derived prostaglandin F2α (PGF2α) (18). This mechanism is thought to trigger the decline of progesterone production and the onset of programmed cell death, called apoptosis, which together ultimately culminate in the elimination of the CL. During regression of the CL, steroidogenic luteal cells (8,23,35,36) and endothelial cells (24) undergo apoptosis. Within the bovine CL, the regulation of apoptosis is thought to be dependent upon immune system components.

Immune cells were first reported within the bovine CL in 1968 (37), and more recently, studies evaluating immune cell populations within the bovine CL have revealed an increase in T lymphocytes and macrophages before the onset of luteolysis (47-49). As immune cells invade the regressing corpus luteum, major histocompatibility complex (MHC) molecules are expressed on luteal cells and are thought to activate immune cells through direct cell-cell signaling. Class I MHC expression increases before the onset of luteolysis (49), as does class II MHC molecules (42,43,45,47,51). Once activated, immune cells secrete signaling peptides called cytokines, such as TNFα, IFNγ, and FasL. TNFα (54,55), IFNγ (47,53,55) and FasL (69,102) mRNA are present in the bovine CL at the time of luteolysis, and it has been proposed that these cytokines exert their effects on the CL by increasing prostaglandin synthesis (72,105), blocking gonadotropin-stimulated steroidogenesis (54,63,67,72) and initiating apoptosis (67,69,96,109)

The Fas receptor (Fas) is a transmembrane protein death receptor in the TNF receptor superfamily (73-75). As Fas ligand (FasL) binds to Fas, apoptosis is induced

through a series of signaling pathways within the cell. The binding of FasL triggers the trimerization of Fas, which recruits Fas associated death domain (FADD) and pro-caspase-8 proteins. The self-cleavage of pro-caspase-8 signals the caspase cascade, which leads to the activation of pro-caspase-3 and apoptosis (82,86). In the regressed bovine CL, Fas and FasL mRNA are present (69,102), implicating this system in luteal cell death. In ovarian cells, the cytotoxicity of FasL is exacerbated by concomitant treatment with TNFα and IFNγ, suggesting these cytokines augment FasL-induced cell death (69,96,109) and regression of the CL (69,101). The Fas-FasL system within the bovine CL may account for the cell-specific death observed during CL regression. That is, during regression of the CL, individual cells undergo apoptosis while neighboring cells remain unaffected (111). In the current study, cytoskeletal components, specifically intermediate filaments, are postulated to influence the expression of Fas on the surface of bovine luteal cells, and hence lend cell specificity to the process of FasL-mediated apoptosis.

The cytoskeleton of cells consists of microtubules, microfilaments, and intermediate filaments. Intermediate filaments have a diameter ranging between 7-11 nm and consist of a family of five different subtypes (112). One of the subtypes is keratin-like proteins, called cytokeratins (CK), which are generally found in epithelial cells. Cytokeratin filaments are obligate heterodimers, forming filaments of an acidic cytokeratin (type I, K9-K20), and a basic cytokeratin (type II, K1-K8) (112,113). The more prominent types of CK filaments found in epithelial cells include filaments containing K7, K8 and K18, K19 (112).

Intermediate filaments have an important role in cell survival. For instance, CK-18 intermediate filaments protect cells from TNFα- and FasL-induced apoptosis. Epithelial cells in which keratin 8 or keratin 18 expression has been genetically knocked-out are considerably more sensitive to TNFα-induced death than their wild-type counterparts (127). In murine hepatocytes, cytokeratin 8/18 intermediate filaments help sequester Fas within the Golgi apparatus, thus impairing Fas expression on the cell surface and protecting the cells from Fas-induced death (85). Taken together, these results suggest cytokeratin intermediate filaments play a role in increasing cell resistance to TNFα- and FasL-induced death.

Intermediate filament expression in the CL of the human (132), rat (133), emu (134) and cow (135,136,137) has been characterized. In the bovine CL, the expression of CK-18 intermediate filaments varies from being highest in early CL to virtually absent in late stage CL (135, 137). These changes in relative expression of CK-18 filaments perhaps reflect functional changes in cell viability and possibly resistance to apoptosis. Thus, further study of them offers potential insight about the function of CK-18 filaments relative to luteal function.

In the present study, the objective was to investigate the potential role of CK-18 intermediate filaments in bovine CL during FasL-induced apoptosis. We hypothesized disruption of CK-18 filaments in luteal cells of bovine CL increases cell surface expression of Fas and susceptibility to FasL-induced apoptosis. Experimentally, cultures of bovine luteal cells from early and late stage CL were exposed acutely to acrylamide to disrupt the CK-18 filaments. The effects of filament disruption on Fas expression and FasL-induced apoptosis were then measured.

Materials and Methods

Corpus Luteum Collection and Dissociation

Estrous cycles of Holstein dairy cows were monitored using transrectal ultrasonography, and corpora lutea (CL) were removed by colpotomy at days 5 (early stage; n=6 cows) and 16-18 (late stage; n=9 cows) post ovulation (ovulation = day 0). Luteal cells obtained from CL at these two stages of luteal function express relatively high and low amounts of cytokeratin intermediate filaments, respectively, based upon previous work (135,137). Prior to CL removal, blood samples were obtained by coccygeal venipuncture using heparinized tubes to measure plasma progesterone concentration by radioimmunoassay and verify the stage of the estrous cycle. Corpora lutea and blood samples were transported to the laboratory on ice where the CL were enzymatically dissociated using collagenase type I (Worthington, Lakewood, NJ) as described previously (138). Briefly, CL were cleaned of connective tissue, weighed, minced, and placed in a spinner flask containing Ham's F12 culture medium with 0.5% bovine serum albumin. The contents of the spinner flask were gently mixed at 37°C in a waterbath. Collagenase was added and the tissue was agitated every 10 minutes for one hour using a wide tip pipette. After the one-hour dissociation, the medium of the spinner flask was decanted into a 50mL tube and fresh collagenase and culture medium were added to the spinner flask for a second, hour-long dissociation. The medium in the 50mL tube from the first dissociation was centrifuged for 10 minutes at 228xg. Afterwards, the supernatant was removed and the cell pellet was resuspended in 50mL Ham's F12 culture medium, followed by successive spins and washes at 129xg and 57xg. After the second dissociation, the pellets

from both dissociations were combined and the luteal cells counted using a hemocytometer. Relative viability of the cells was determined by trypan blue exclusion. The cells were then either fixed in paraformaldehyde for flow cytometric analysis of cytokeratin and Fas expression, or placed in culture for further experimentation (see below).

For the immediate fixation of luteal cells, 0.3mL of $1.5x10^6$ freshly dissociated viable cells added to 0.1mL Ham's F12 culture medium was centrifuged using screen-capped tubes (Ref # 352235, BD Falcon, San Jose, CA) for 5 minutes at 276xg, 4°C. The filtered cells were then fixed for 2 hours on ice by adding 0.4mL 2% paraformaldehyde to the 0.4mL cell suspension for a final concentration of 1% paraformaldehyde. After fixation, the cells either remained in fixative or were rinsed twice with 1x PBS and permeabilized using 70% ethanol (EtOH). Both the fresh-fixed and permeabilized cells were stored at 4°C (those held in fixative) or 20°C (those held in EtOH) until further processed for flow cytometry.

For cell culture experiments, freshly dissociated luteal cells were seeded in T25 flasks at a density of $2x10^6$and in 8-well microchamber slides at $2x10^5$ viable cells/well . The cells were cultured in Ham's F12 culture medium (Invitrogen, Carlsbad, CA) supplemented with insulin, transferrin, selenium (ITS; 5µg/5µg/5ng/mL; Sigma Aldrich, St. Louis, MO) and gentamicin (20µg/mL; Invitrogen [Gibco], Carlsbad, CA) and incubated at 37°C, 5% CO_2 in air and 95% humidity overnight. The day after seeding, the flasks and chamber slides were rinsed and the conditioned medium replaced with

fresh culture medium prior to treatments. Initially, flasks and chamber slides were treated with either culture medium (control) or 5mM acrylamide (Fisher Scientific, Pittsburgh, PA) for 4 hours to disrupt intermediate filaments. After this initial treatment period, all flasks and chamber slides were rinsed twice and the medium replaced. Cells from several flasks were immediately trypsinized using trypsin-EDTA (Cell Gro Mediatech, Manassas, VA) for flow cytometric analysis of cytokeratin and Fas expression. Briefly, the flasks were rinsed 2 x 5 minutes with Hank's Balanced Salt Solution (Sigma Aldrich, St. Louis, MO), followed by two quick washes with trypsin-EDTA. After the second rinse, the remaining trypsin was removed and the flasks were left un-touched for 10 minutes. Following this 10-minute incubation, the cells were collected in Ham's F12 culture medium containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), centrifuged for 5 minutes at 276xg, 4°C, and then fixed as described above. The remaining flasks were treated with a cytokine cocktail containing bovine interferon-γ (IFN, 200 IU/mL; R&D Systems, Minneapolis, MN), murine tumor necrosis factor-α (TNF, 10ng/mL; US Biological, Swampscott, MA), human recombinant soluble Fas ligand (FasL, 50ng/mL; R&D Systems, Minneapolis, MN), and murine monoclonal anti-6x histidine cross-linking antibody (1mg/mL; R&D Systems, Minneapolis, MN) for 24 hours to induce cell death. After 24 hour incubation, the flasks were trypsinized and fixed as described above or re-treated with the cytokine cocktail for an additional 24 hours, then trypsinized and fixed. Conditioned medium was saved from all flasks prior to fixation for progesterone analysis.

Cells in microchamber slides were used to evaluate microscopically the efficacy of acrylamide as disrupter of cytokeratin intermediate filaments. The cells were fixed using 4% paraformaldehyde in 1x PBS. Briefly, the chamber slides were rinsed twice with 1x PBS, fixed at room temperature for 20 minutes, and then stored in 1x PBS at 4°C until analyzed by fluorescent microscopy.

The heparinized blood samples from the cows were centrifuged at 2056xg for 20 minutes at 4°C to obtain plasma, which was then frozen until progesterone analysis.

Immunodetection of Cytokeratin 18-Containing Intermediate Filaments and Microtubules

Chamber slides containing the previously-fixed luteal cells were rinsed twice with PBS containing 0.1% bovine serum albumin (PBS-BSA) followed by a 1 hour block/permeabilization step with 0.3% triton x-100 in 1x PBS containing 10% normal goat serum (Vector Labs, Burlingame, CA) and 3% BSA. The slides were rinsed 3 x 5 minutes with PBS-BSA and incubated overnight at 4°C with either a mouse anti-human CK-18 monoclonal antibody (clone CY-90; Sigma Aldrich, St. Louis, MO; diluted 1:800 in PBS-BSA with 10% normal goat serum), or a mouse anti-bovine alpha tubulin monoclonal antibody (clone 236-10501; Invitrogen, Carlsbad, CA; diluted 1:200 in PBS-BSA with 10% normal goat serum). The following day, after 3 x 5 minute washes with PBS-BSA, fluorescent detection of the CK18-containing filaments or tubulin-containing microtubules was achieved by incubating the slides with a goat anti-mouse Alexa 488-conjugated IgG antibody (CK18; Invitrogen, Carlsbad, CA) or a goat anti-mouse Texas

Red-conjugated antibody (Microtubules; Santa Cruz, Santa Cruz, CA). Both secondary antibodies were diluted 1:200 in PBS-BSA with 10% normal goat serum (Vector Labs, Burlingame, CA). The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Vector, Burlingame, CA) and then coverslipped.

Cell Death Counts

Cell death was assessed at three different times during the experiment. The number of attached luteal cells in five random microscopic fields of view was counted in all of the flasks prior to cytokine treatment using a 0.25mm^2 grid (initial cell counts). At 24 and 48 hours after treatment, the number of attached cells in the flasks was again counted to estimate cell loss (post treatment cell counts). All five fields of view per flask were averaged and the percent cell death was determined using the following equation:

$$\% \text{ Cell Death} = [1-(\text{Post treatment cell counts} / \text{initial cell counts})] * 100$$

Flow Cytometric Quantification of Fas and CK18 Expression

Freshly dissociated and luteal cells from cultures were washed 2 x 5 min with 1x PBS-BSA and centrifuged at 276xg for 5 minutes at 4°C between each wash. Following the second wash, the cells were stained for Fas using a mouse anti-human Fas antibody (clone CH11; Millipore, Billerica, MA; diluted 1:25 with PBS with 10% normal goat serum). The cells were incubated in primary antibody overnight at 4°C and then washed 2 x 5 min with 1x PBS-BSA with spins at 276xg for 5 minutes at 4°C between each wash. Detection of the primary antibody was achieved fluorescently using a goat anti-

mouse Alexa 488-conjugated IgG secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:200 with PBS-BSA with 10% normal goat serum. For detection of CK18, both freshly dissociated and cultured luteal cells were washed 2 x 5 minutes with 1x PBS-BSA and spun at 276xg for 5 minutes at 4°C between each wash. The cells were then incubated for 1 hour at 37°C with a mouse anti-human CK18 FITC-conjugated antibody (clone CY-90; Sigma Aldrich, St. Louis, MO; diluted 1:100 with PBS- BSA). Quantification of cells expressing Fas and CK18 was accomplished using a 4 color, dual laser FACScalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) with a 488nm argon laser for FITC/Alexa 488 excitation. Data were collected using Cell Quest (Becton Dickinson Biosciences, San Jose, CA) and graphs of the results were generated using WinMDI 2.9 software (Scripps Institute, La Jolla, CA). Relative mean fluorescence intensity (MFI) was calculated using the following equation:

MFI=(Geometric Mean Sample – Geometric Mean neg CTL) / Geometric Mean neg CTL

Progesterone Measurement in Plasma and Conditioned Culture Medium

Plasma samples were analyzed for progesterone by radioimmunoassay (RIA) as described previously (139). Briefly, the plasma was extracted twice with 10 volumes of petroleum ether. The petroleum ether was then evaporated and the samples reconstituted in PBS with gelatin. Progesterone was determined by RIA using anti-progesterone-11-BSA serum. Progesterone concentrations were corrected for recovery.

Similarly, progesterone concentration in the conditioned medium from all flasks was measured by radioimmunoassay as described previously (140). Once collected, the conditioned medium was spun at 276xg, and the supernatant was saved and stored at -20°C until analysis. Progesterone was detected using anti-progesterone-11-BSA serum (final dilution was 1:10,000). After charcoal adsorption, the supernatant was placed into vials and Ready Safe Cocktail was added. Radioactivity was determined in a liquid scintillation counter. Progesterone concentrations were normalized to $5x10^5$ cells.

Progesterone Measurement in Cell Fractions

An unexpected observation in the current study was that progesterone in the conditioned medium of cells undergoing apoptosis was higher than that of controls. There are two possible explanations for this: 1) Cell death results in greater synthesis of progesterone, or 2) As cells die and break open, a greater amount of progesterone is released into the culture medium. To address this issue the amount of progesterone secreted by live cells versus dying cells was calculated on a per-DNA basis. Death was induced in luteal cultures using 5mM acrylamide for 24 and 48 hours. Following treatment, the conditioned medium was spun at 276xg and the supernatant was collected and stored at -20°C for later analysis. The cell pellet (dead cell fraction) was similarly stored at -20°C for progesterone analysis and DNA extraction. Lastly, the attached cells within the flasks (live cell fraction) were trypsinized and stored at -20°C until progesterone analysis and DNA extraction. Progesterone was extracted from both dead and live cell fractions as described for plasma progesterone extraction, and progesterone secretion in the conditioned medium was determined as described above. Following progesterone

extraction, the dead and live cell fractions were also subjected to DNA extraction. All samples were reconstituted in Tris/sodium chloride/EDTA buffer (TNE), to a final volume on 0.6mL. Trichloroacetic acid (TCA; 40%) was added to all samples for a final concentration of 10% TCA. The samples were incubated on ice for 10 minutes and then centrifuged at 1428xg. The supernatants were discarded and the pellets were resuspended in 10% TCA to a final volume of 0.6mL. The samples were placed in a water bath at 95°C for 30 minutes, followed by 5 minutes to cool on the bench top, and then 5 minutes on ice. The samples were then centrifuged at 1428xg and the supernatant collected and analyzed for DNA content. For the DNA assay, all samples were reconstituted in 1mL TNE. Diphenylamine reagent was added to all samples and mixed. Next, all samples were placed in a water bath at 95°C for 10 minutes, followed by 5 minutes to cool on the bench top. Absorbance was measured using Spectronic Genesys 5 at 600nm and the DNA content of the samples was determined using a standard curve. Progesterone concentrations for the dead and live cell fractions were then corrected for DNA content.

HepG2 Culture

Murine hepatocytes were among the first cells in which cytokeratin intermediate filaments were implicated in Fas trafficking (85). Considering this, the current study utilized hepatocyte carcinoma cells (HepG2 cells) as a positive control cell type to determine if acrylamide disrupts intermediate filaments and alters cell surface expression of Fas. HepG2 cells were seeded into T150 flasks at $2x10^6$ cells per flask. For each experiment, two vials ($1x10^6$ cells per vial) at the same passage were placed in culture.

The cells were cultured in EMEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) and incubated at 37°C, 5% CO_2 and 95% humidity. At approximately 70% confluency, the HepG2 cells were passaged into T25 flasks. The cells were washed twice for 5 minutes with Hank's Balanced Salt Solution (Sigma Aldrich, St. Louis, MO), followed by two quick washes with trypsin-EDTA (Cell Gro Mediatech, Manassas, VA). After the second rinse with trypsin-EDTA, the remaining trypsin was removed and the flask was left untouched for 10 minutes. The cells were then collected in EMEM with 10% FBS and centrifuged at 112xg for 5 minutes at 4°C. Numbers of viable cells were determined using trypan blue exclusion (Sigma Aldrich, St. Louis, MO) and the cell pellet was divided among six T25 flasks at approximately $1x10^6$ cells per flask. The following day, the medium was exchanged and the cultures were exposed to vehicle (control) or 5mM acrylamide for 4 hours. Following treatment, the cultures were fixed with 2% paraformaldehyde in 1x PBS and processed for flow cytometry to assess cell surface expression of Fas expression as described above. These experiments were repeated a total of 4 times for each stage of CL.

Statistical Analysis

The data were analyzed by 1-way or 2-way ANOVA followed by Tukey's multiple comparison test in the general linear model of Systat 12.0 (Point Richmond, CA). Results are expressed as mean $\pm$ SEM and a value of $P<0.05$ was considered significant.

Results

Characterization of CL Collected

Early stage CL were smaller than late stage CL (3.5 ± 0.35 vs. 6.3 ± 0.7 grams; $P<0.05$). Similarly, systemic concentrations of progesterone for the cows differed ($P<0.05$), being lower for early stage cows compared to late stage cows.

Expression of CK-18 Intermediate Filaments in Freshly Isolated Bovine Luteal Cells

Flow cytometric analysis of CK-18 expression revealed greater numbers of CK-18 fluorescent luteal cells of early stage CL compared to late stage CL ($P<0.05$; Fig. 7). A two-fold decrease in numbers of CK-18 luteal cells occurred as the tissue transitioned from early to late stage CL (Fig. 7b).

Expression of Fas on Freshly Isolated Bovine Luteal Cells

The number of luteal cells expressing Fas at the cell surface was higher for early stage CL than late stage CL ($P<0.05$; Fig. 8a,b). Relative mean fluorescence, a measure of fluorescent staining intensity on the cells, was higher for early stage CL than late stage CL ($P<0.05$; Fig. 8c). Similar results were seen for the two stages of CL when the total amount of Fas (i.e., both cell surface and intracellular Fas) was analyzed ($P<0.05$; Fig. 9).

Effects of Acrylamide on CK-18 Intermediate Filaments, Microtubules, and Progesterone Secretion

Acrylamide effectively disrupted CK-18 intermediate filaments in cultured bovine luteal cells (Fig. 10). Control cultures exhibited extensive, filamentous networks of CK18

staining (Fig. 10a) that became aggregated and punctate following acrylamide exposure (Fig. 10c). Conversely, there was no effect of acrylamide on microtubule organization when control and acrylamide-treated cultures were compared (Figs. 10b and 10d, respectively), verifying the specificity of acrylamide to intermediate filaments. Flow cytometric analysis revealed that despite the disruption of CK-18 filament organization, acrylamide did not reduce the overall number of cells expressing CK-18 ($P>0.05$; Fig. 11). Lastly, acrylamide had no effect on progesterone secretion by the cultures ($P>0.05$; Fig. 12), indicating there was no adverse effects on cell viability.

As a positive control, HepG2 cells were utilized to assess the effect of acrylamide on CK-18 intermediate filaments and microtubules (Fig. 13). Acrylamide disrupted CK-18 filament expression as well as disrupted microtubule organization in HepG2 cells (Fig. 13c,d). CK-18 filaments and microtubules were aggregated around the nucleus, no longer displaying a filamentous network as seen in control cultures. However, the structural organization of CK-18 intermediate filaments and microtubules fully recovered within 24 hours of removing acrylamide from the culture medium (data not shown), indicating that acrylamide treatment was not inducing death in HepG2 cells.

Effects of Acrylamide-induced CK-18 Filament Disruption on Fas Surface Expression

Although acrylamide effectively disrupted CK-18 filaments, its transient actions failed to enhance the number of cells expressing Fas at the cell surface ($P>0.05$; Fig. 14a,b,c). Moreover, CK18 filament disruption failed to enhance Fas cell surface expression in

specific cells, as reflected by the lack of change in relative mean fluorescence intensity ($P>0.05$; Fig. 14d). Consistent with the observations of freshly isolated luteal cells, cultured luteal cells of early stage CL expressed higher amounts of Fas on the surface than cultured cells of late stage CL ($P<0.05$, Fig. 14c,d).

The disruption of CK-18 intermediate filaments and microtubules by acrylamide resulted in slight (Fig. 15a) to pronounced (Fig. 15b,c) changes in surface expression of Fas on HepG2 cells. Averaging all three experiments revealed that acrylamide increased the number of cells expressing Fas at the cell surface ($P<0.05$; Fig. 16a), but did not increase the mean fluorescence intensity ($P>0.05$; Fig. 16b).

Effects of CK-18 Filament Disruption on Cytokine-induced Fas Expression, Cell Death, CK-18 Intermediate Filaments, and Progesterone Secretion

After 24 hour cytokine treatment, Fas expression remained unchanged for early stage and late stage cultured bovine luteal cells ($P>0.05$; Fig. 17a). The relative mean fluorescence intensity also remained unchanged by cytokine treatment for both early and late stage cultured bovine luteal cells ($P>0.05$; Fig. 17b). Similar results were seen after 48 hour cytokine treatment ($P>0.05$, Fig. 18).

Visual observations revealed that after 48 hour cytokine treatment, cell viability was decreased in cytokine-treated cultures for both early (Fig. 19) and late (Fig. 20) stage CL. Cytokine-treated cultures contained substantial cell death as evidenced by numerous floating cells (Fig. 19c,d, Fig. 20c,d). A quantitative assessment confirmed visual

observations, indicating that cytokine-treatment decreased cell viability ($P<0.05$; Fig. 21, Fig. 22), and CK-18 filament disruption via acrylamide did not enhance this effect ($P>0.05$; Fig. 21, Fig. 22).

Flow cytometric analysis revealed that 24 hour cytokine treatment did not alter cytokeratin-18 expression as compared to control in either early or late stage cultured bovine luteal cells ($P>0.05$; Fig. 23a). Similar results were obtained after 48 hour cytokine treatment (Fig. 23b).

Progesterone secretion by cultures were measured after 24 and 48 hours of cytokine treatment. After 24 hours of cytokine exposure, there was no effect on progesterone secretion by early or late stage cultured luteal cells ($P>0.05$; Fig. 24a). However, after 48 hours of cytokine exposure, progesterone secretion decreased compared to acrylamide-treated cultures for early stage cultured luteal cells ($P<0.05$; Fig. 24b). There was no effect of 48 hour cytokine exposure on progesterone secretion by late stage cultured luteal cells ($P>0.05$; Fig. 24b).

Progesterone content was measured for live and dead cell fractions and conditioned medium after 24 and 48 hours of acrylamide treatment. Progesterone content from dead cell fractions tended to be higher than the corresponding live cell fractions (Fig. 25a). The amount of progesterone in the dead cell fraction was highest in cultures exposed to acrylamide for 48 hours (Fig. 25a). Secretion of progesterone in 24 and 48 hour

acrylamide-treated cultures did not appear to change compared to the control cultures (Fig. 25b).

Figure 7: Expression of cytokeratin-18 intermediate filaments in early and late stage freshly isolated bovine luteal cells as determined by flow cytometric analysis.

Figure 7a:

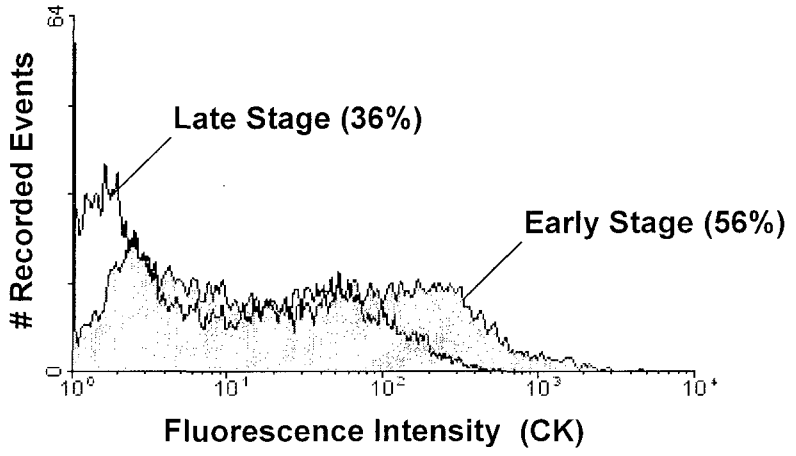
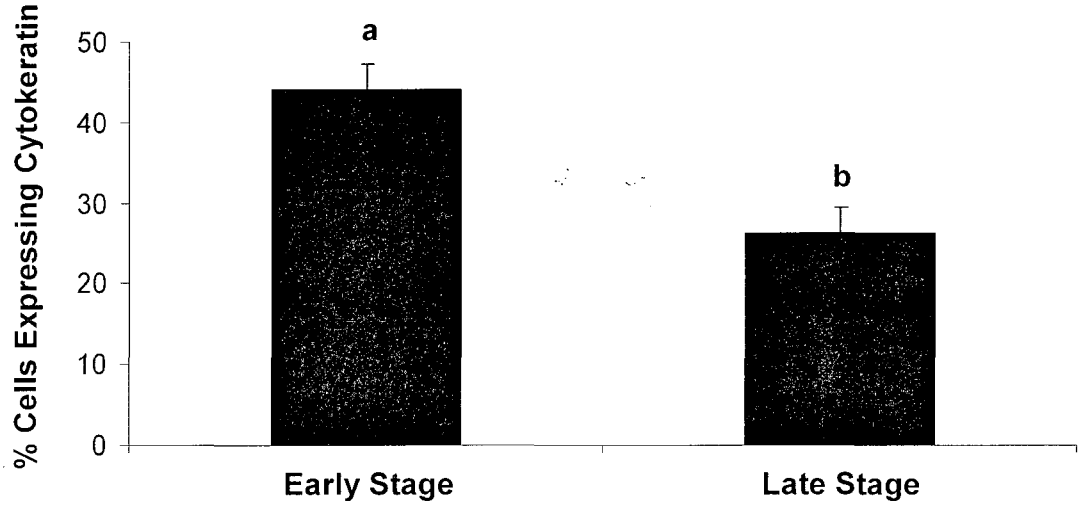


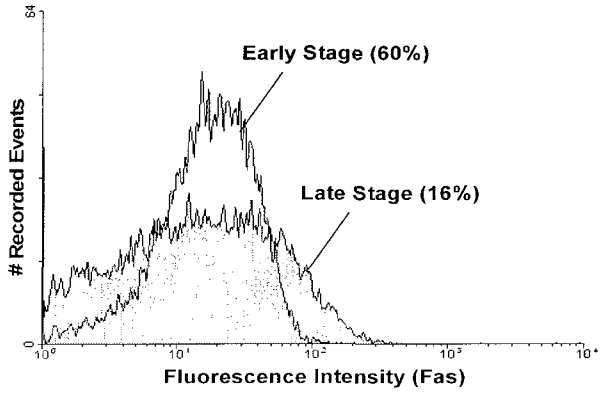
Figure 7b:



A representative flow cytometric histogram depicting the detection of cytokeratin-18 intermediate filaments within early and late stage bovine luteal cells (Fig. 7a). The relative number of cells expressing cytokeratin-18 intermediate filaments was higher for early stage CL than late stage CL (Fig. 7b). Values shown are mean $\pm$ SEM; different letters indicate significant differences ($P<0.05$; n=6-9 CL/stage).

Figure 8: Expression of Fas on the surface of early and late stage freshly isolated bovine luteal cells as determined by flow cytometric analysis.

Figure 8a:



A representative flow cytometric histogram depicting the detection of Fas on the surface of freshly isolated bovine luteal cells (Fig. 8a). Relative number of cells expressing Fas at the membrane surface was higher for early vs. late stage CL (Fig. 8b). Relative mean fluorescence intensity (MFI) was higher for early stage CL than late stage CL (Fig. 8c). Values shown are mean ± SEM; different letters indicate significant differences ($P<0.05$; n=6-9 CL/stage).

Figure 8b:

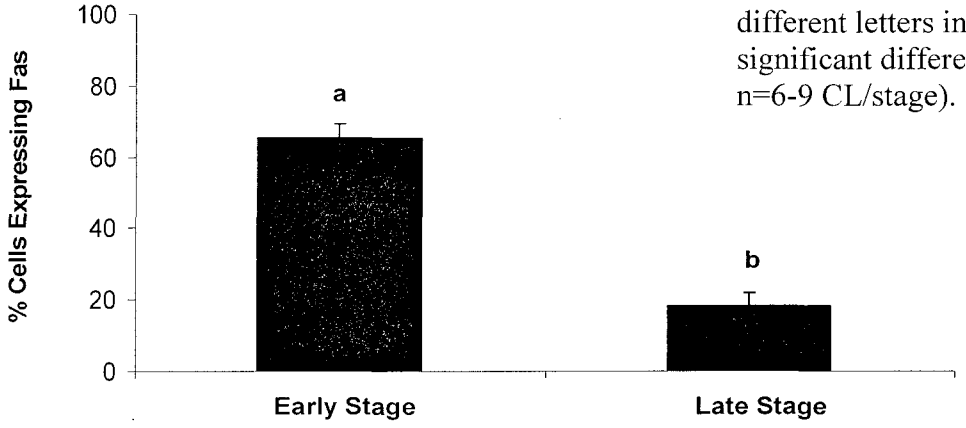


Figure 8c:

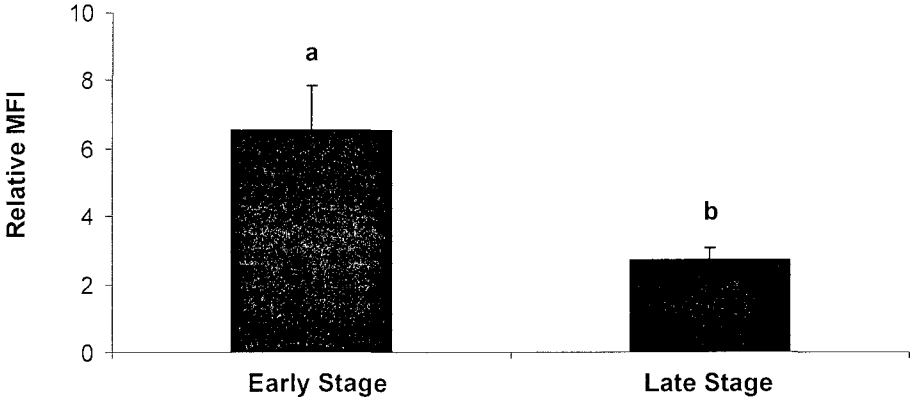
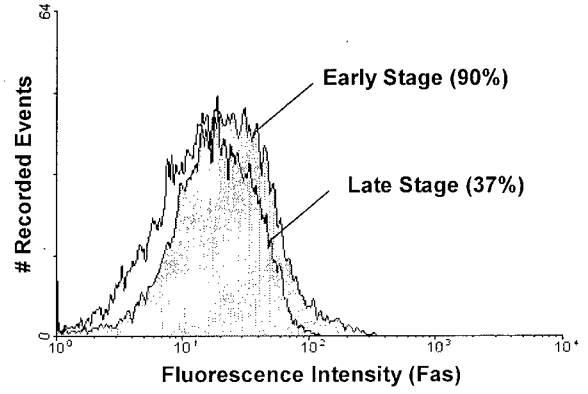


Figure 9: Total expression of Fas within early and late stage freshly isolated bovine luteal cells as determined by flow cytometric analysis

Figure 9a:



A representative flow cytometric histogram depicting the total amount of Fas detected within freshly isolated bovine luteal cells (Fig. 9a). Relative number of cells expressing Fas was higher for early vs. late stage CL (Fig. 9b). Relative mean fluorescence intensity (MFI) was higher for early stage CL than late stage CL (Fig. 9c). Values shown are mean ± SEM; different letters indicate significant differences ($P<0.05$; n=4-9 CL/stage).

Figure 9b:

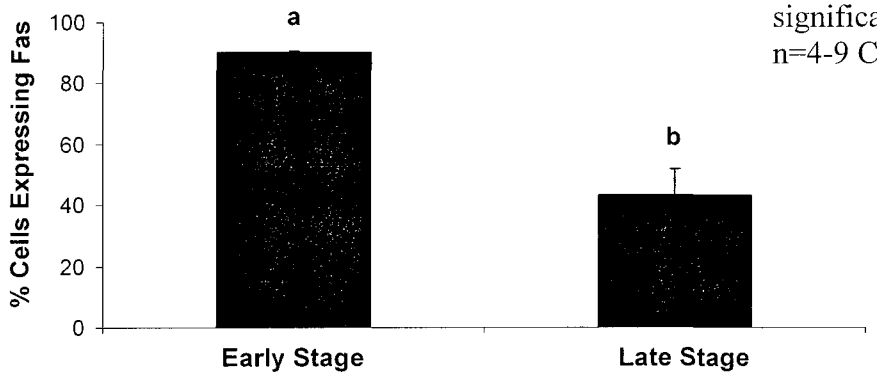


Figure 9c:

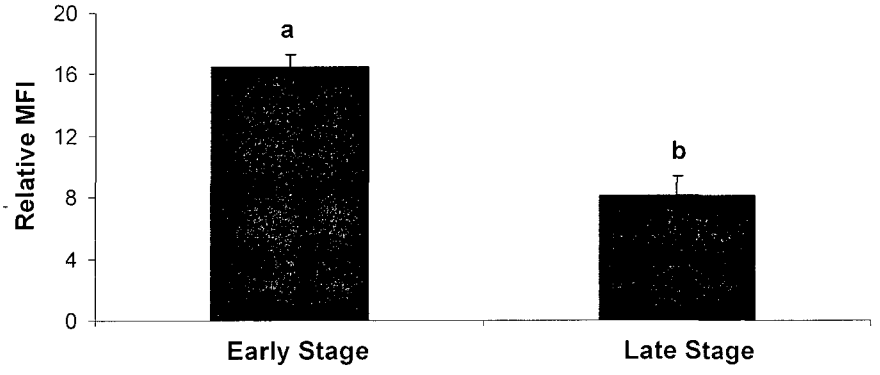
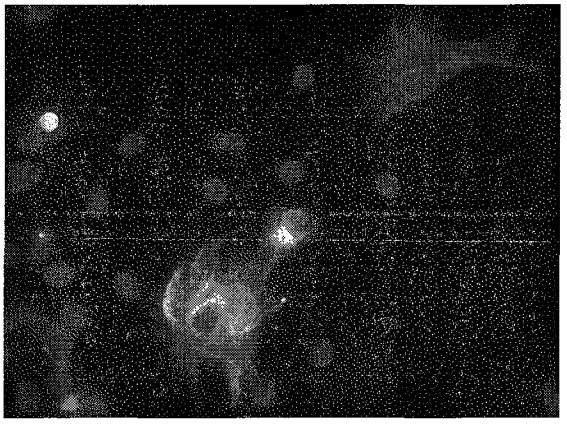


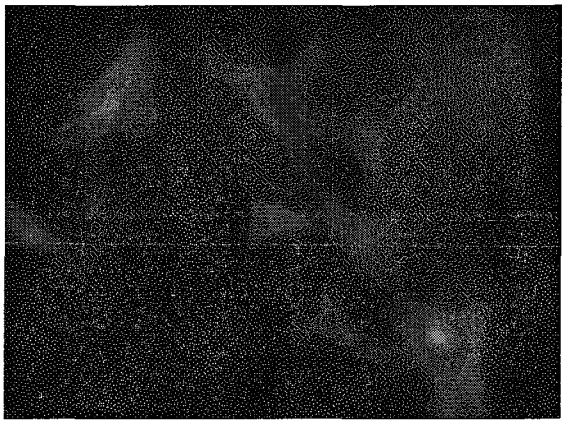
Figure 10: Effects of acrylamide on cytokeratin-18 (CK-18) intermediate filaments and microtubules (MT).

Figure 10a:



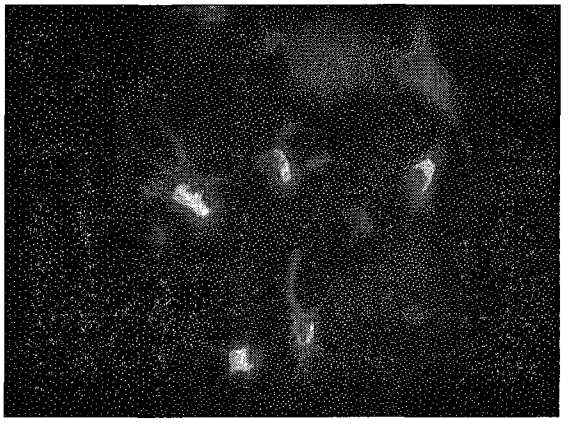
Control (CK-18)

Figure 10b:



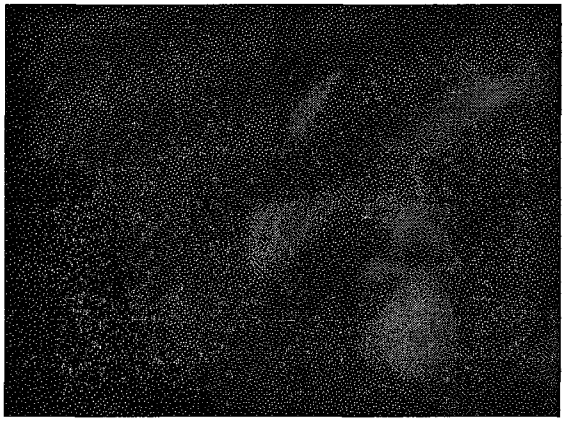
Control (MT)

Figure 10c:



Acrylamide (CK-18)

Figure 10d:



Acrylamide (MT)

Fluorescent detection of cytokeratin-18 filaments (Fig. 10a,c, green fluorescence) and microtubules (Fig. 10b,d, red fluorescence) in control (Fig. 10a,b) and acrylamide-treated (Fig. 10c,d) cultures. Control cells displayed a filamentous cytoskeleton, which spanned the cytoplasm (Fig. 10a,b). Acrylamide-treated cultures exhibited disrupted cytokeratin-18 intermediate filaments with peri-nuclear aggregation (Fig. 10c). Microtubules were unaffected by acrylamide treatment (Fig. 10d). Magnification: 40x.

Figure 11: Effect of acrylamide on cytokeratin-18 intermediate filament expression as determined by flow cytometric analysis.

Figure 11a:

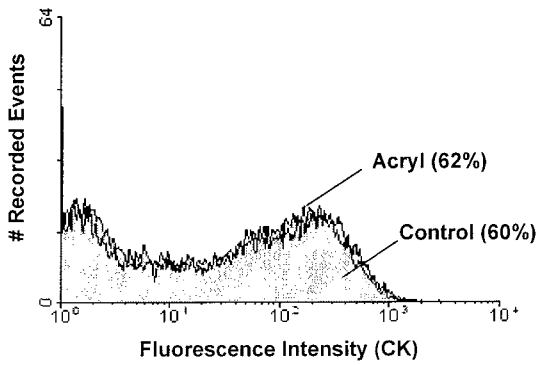
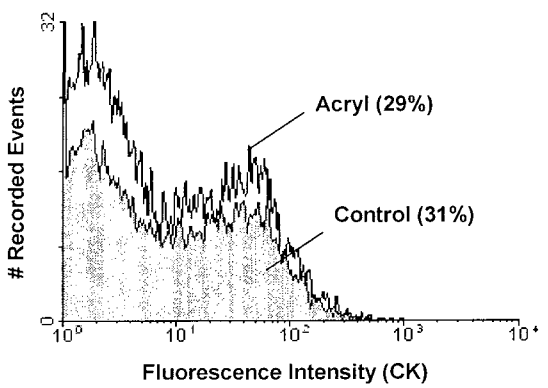


Figure 11b:



Representative flow cytometric histogram depicting the effect of acrylamide on cytokeratin-18 expression in early stage (Fig. 11a) and late stage (Fig. 11b) CL. There was no effect on the number of cells expressing cytokeratin for either stage (Fig. 11c). Different letters indicate significant differences ($P<0.05$; n=2 CL/stage).

Figure 11c:

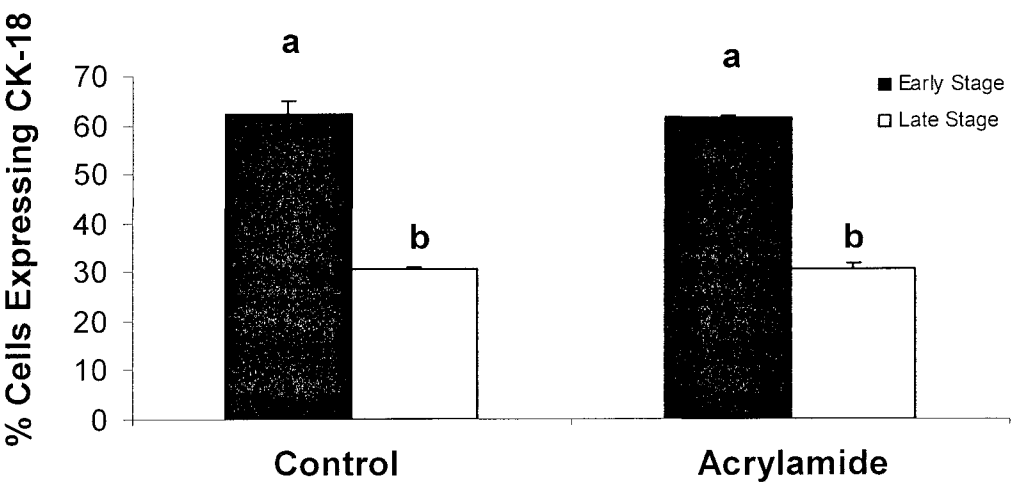
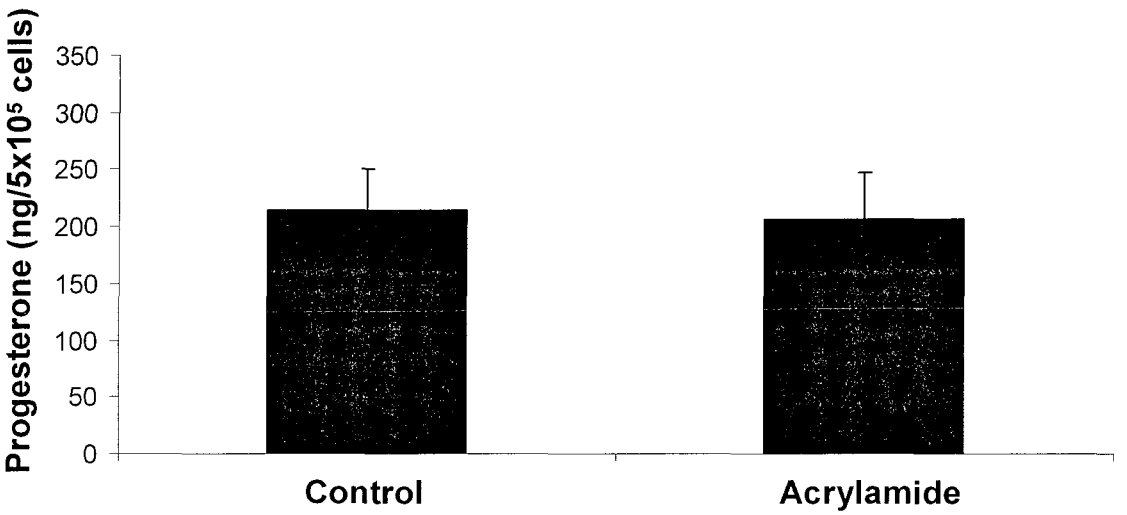


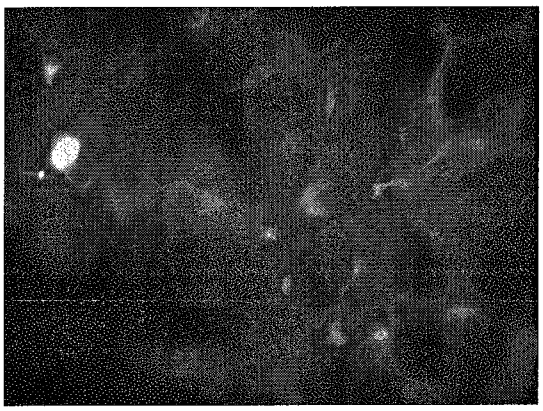
Figure 12: Effect of acrylamide on progesterone secretion by cultured bovine luteal cells.



Progesterone secretion by cultured bovine luteal cells remained unchanged after 4 hours of acrylamide treatment. No effect of stage (i.e., early vs. late stage CL) was observed ($P>0.05$, n=4 CL/stage), thus the results of the two stages were pooled (mean $\pm$ SEM).

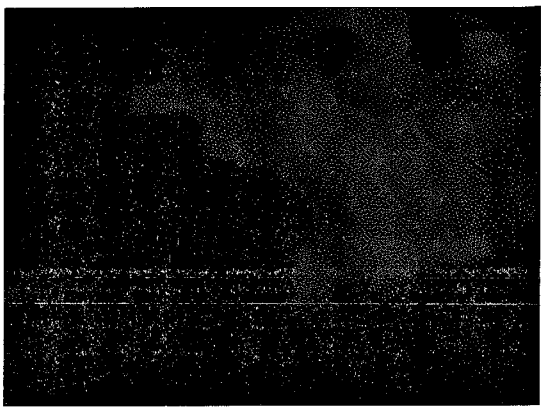
Figure 13: Effects of acrylamide on cytokeratin-18 (CK-18) intermediate filaments and microtubules (MT) in HepG2 cells.

Figure 13a:



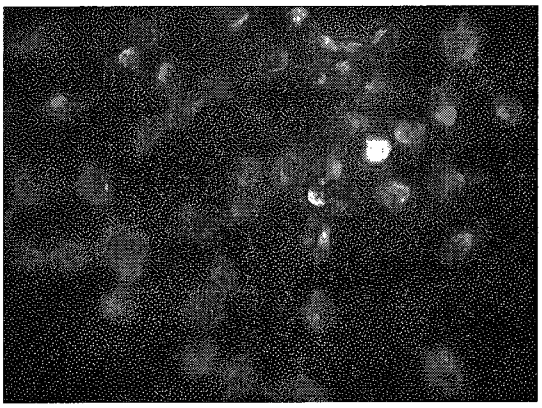
Control (CK-18)

Figure 13b:



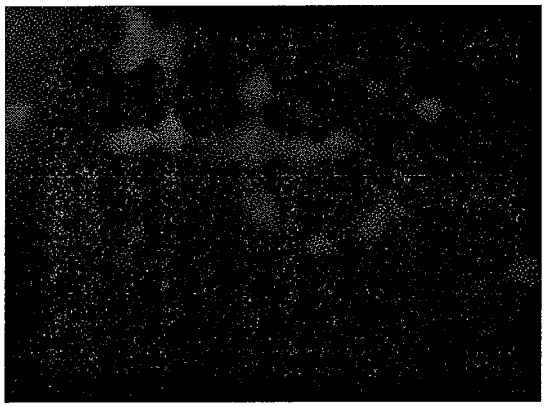
Control (MT)

Figure 13c:



Acrylamide (CK-18)

Figure 13d:



Acrylamide (MT)

Fluorescent detection of cytokeratin-18 filaments (Fig. 13a,c, green fluorescence) and microtubules (Fig. 13b,d, red fluorescence) in control (Fig. 13a,b) and acrylamide-treated (Fig. 13c,d) cultures. Control cells displayed a filamentous cytoskeleton, which spanned the cytoplasm (Fig. 13a,b). Acrylamide-treated cultures exhibited both disrupted cytokeratin-18 intermediate filaments and microtubules with peri-nuclear aggregation (Fig. 13c). Magnification: 40x.

Figure 14: Effects of acrylamide on the surface expression of Fas on cultured bovine luteal cells as determined by flow cytometric analysis.

Figure 14a:

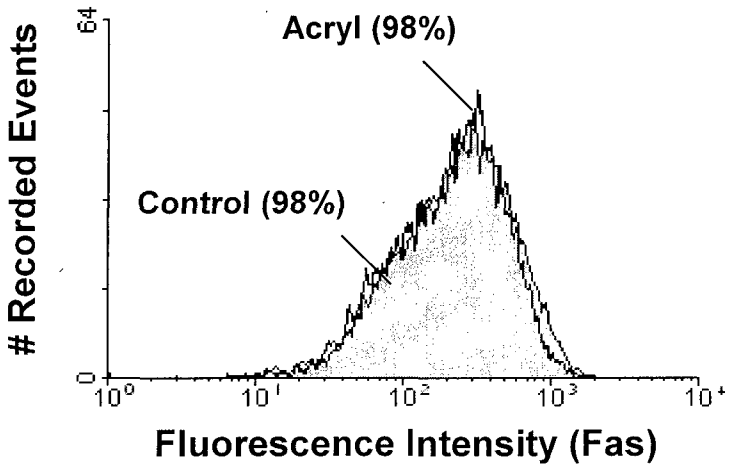
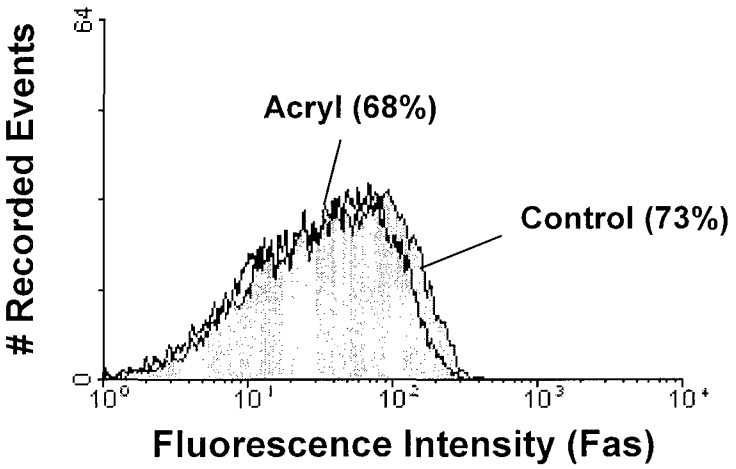


Figure 14b:



Representative flow cytometric histograms depicting the effect of acrylamide on the surface expression of Fas on cultured bovine luteal cells. No effect of acrylamide was seen for early stage CL (Fig. 14a) or late stage CL (Fig. 14b).

Figure 14c:

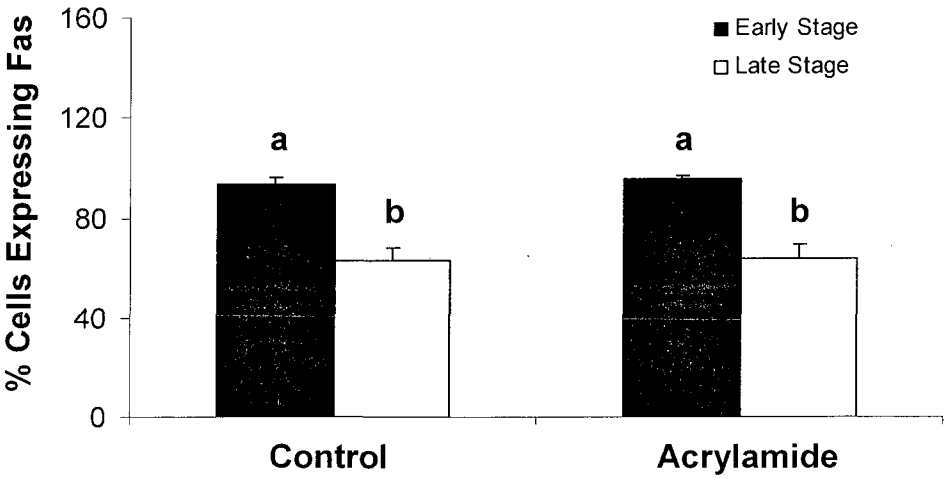
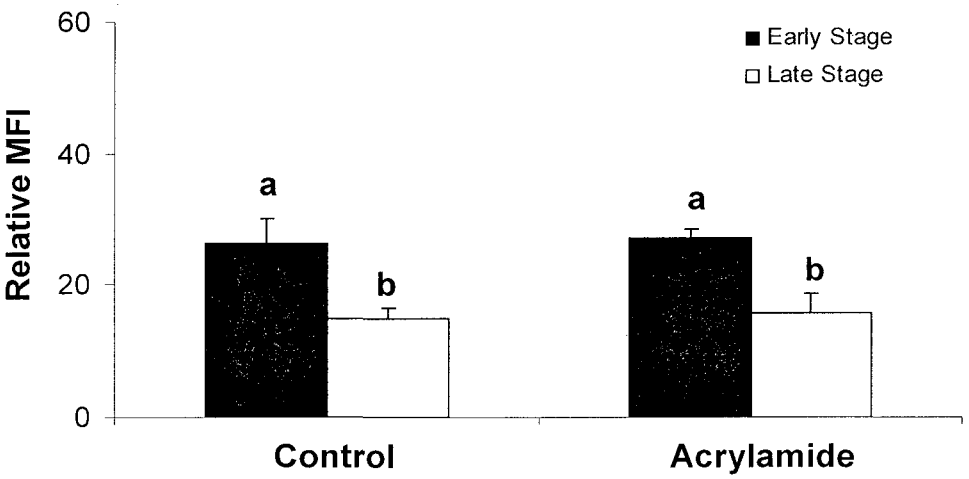


Figure 14d



Effect of acrylamide on the surface expression of Fas (mean $\pm$ SEM) on bovine luteal cells. The percentage of Fas-positive cells (Fig. 14c) and mean fluorescence intensity of Fas expression (Fig. 14d) are depicted. Different letters indicate significant differences ($P<0.05$; n=4 CL/stage).

Figure 15: Effect of acrylamide on surface expression of Fas on HepG2 cells as detected by flow cytometric analysis.

Figure 15a:

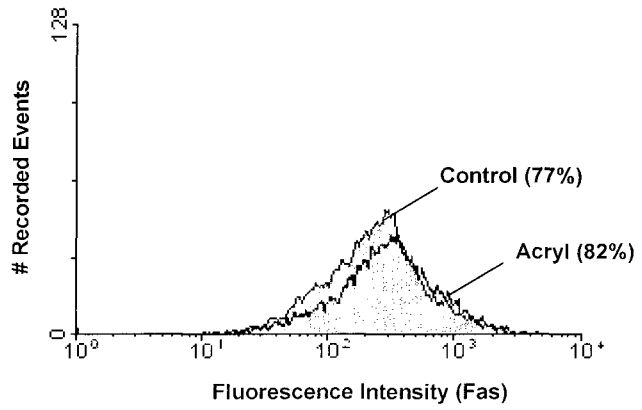


Figure 15b:

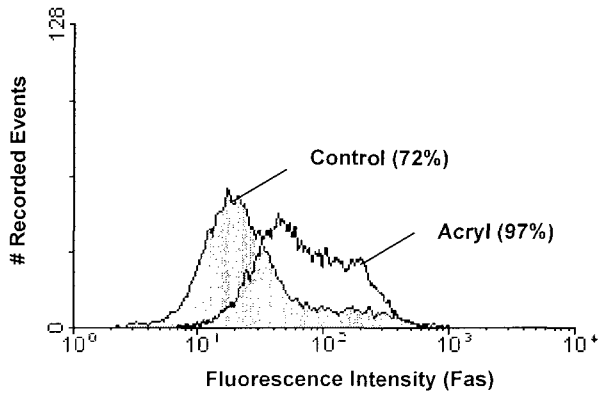
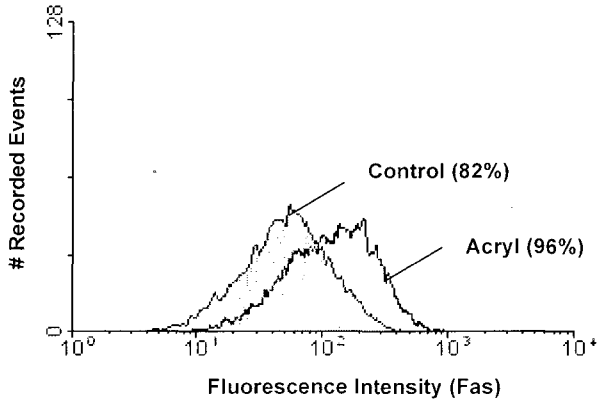


Figure 15c:



Representative histograms from three separate experiments depicting the effect of acrylamide on the surface expression of Fas on HepG2 cells. Modest (5%) to pronounced (24%) shifts in Fas expression were seen as a result of acrylamide treatment in all three experiments (Fig. 15).

Figure 16: Effect of acrylamide on the surface expression of Fas on HepG2 cells.

Figure 16a:

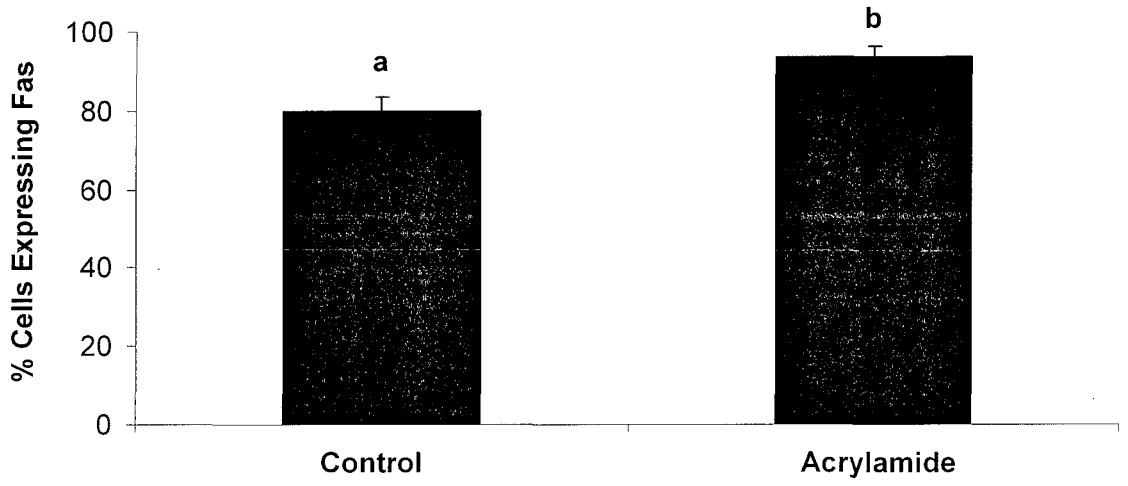
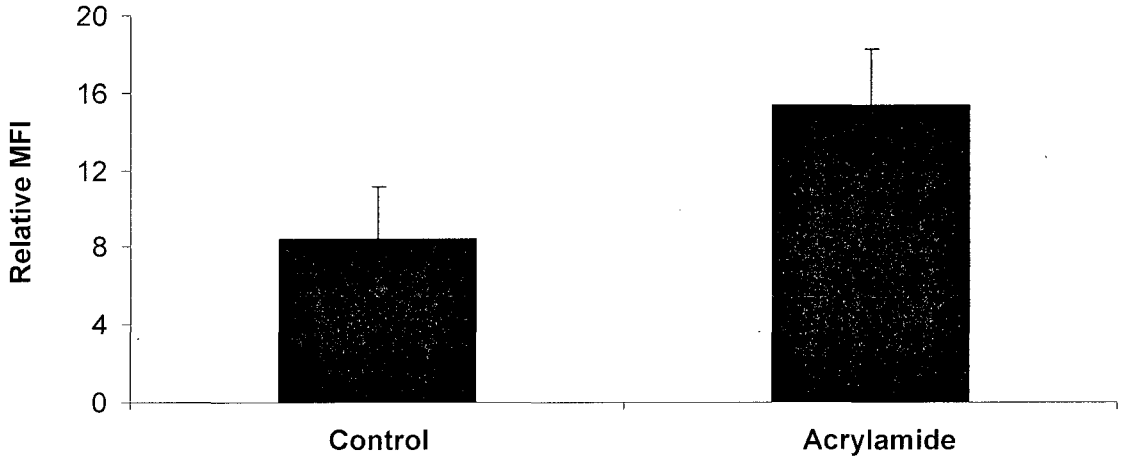


Figure 16b:



The effect of acrylamide on the percentage of HepG2 cells expressing Fas and the relative mean fluorescence intensity (MFI) of Fas expression (mean $\pm$ SEM). Acrylamide enhanced the relative number of cells expressing Fas on the surface (Fig. 16a), but did not alter the mean fluorescence intensity (Fig. 16b). Different letters indicate significant differences ($P<0.05$; n=3 experiments).

Figure 17: Effects of 24 hour cytokine treatment on the surface expression of Fas on cultured bovine luteal cells as determined by flow cytometric analysis.

Figure 17a:

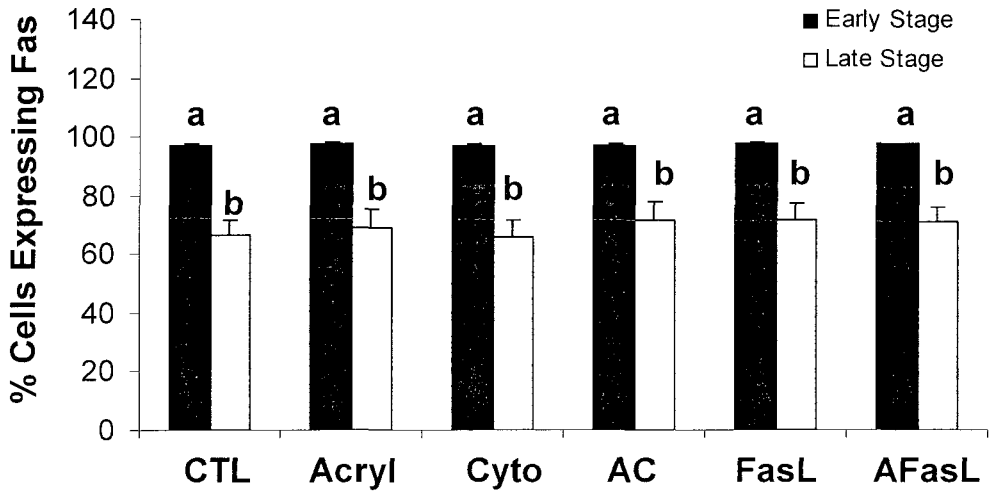
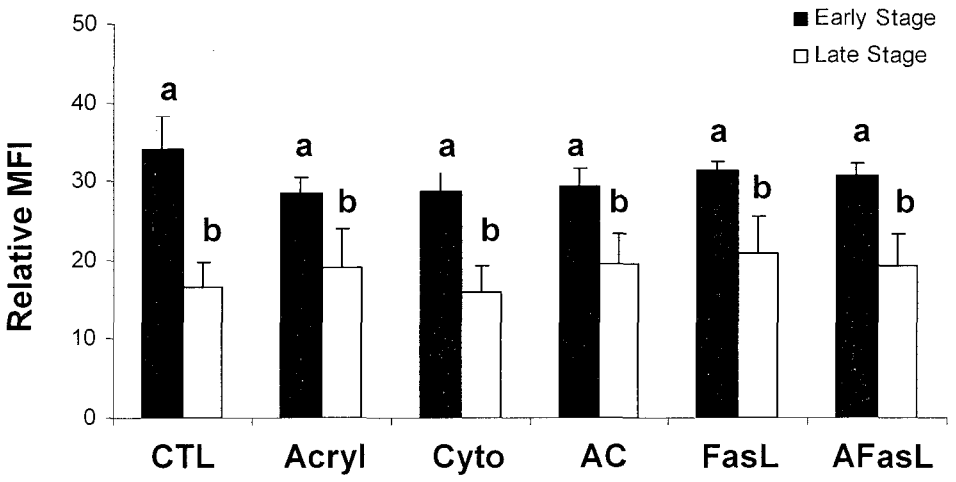


Figure 17b:



The effect of 24 hour cytokine-treatment on Fas expression (mean ± SEM) on the surface of cultured luteal cells from early and late stage bovine CL (Fig. 17). Treatment did not alter the number of cells expressing Fas (Fig. 17a), or the relative mean fluorescence intensity (Fig. 17b). Different letters indicate significant differences ($P<0.05$; $n=4$ CL/stage). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines, FasL = Fas Ligand, AFasL = Acrylamide + Fas Ligand)

Figure 18: Effect of 48 hour cytokine treatment on the surface expression of Fas on cultured bovine luteal cells as determined by flow cytometric analysis.

Figure 18a:

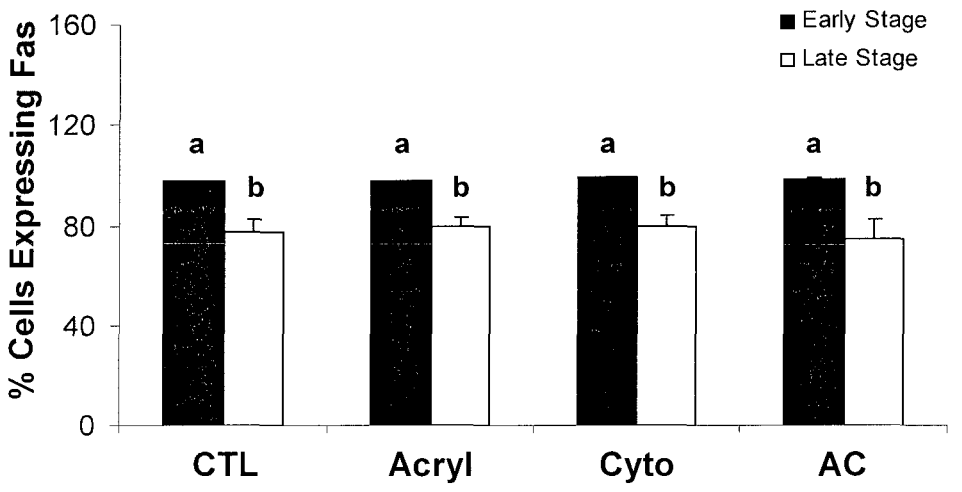
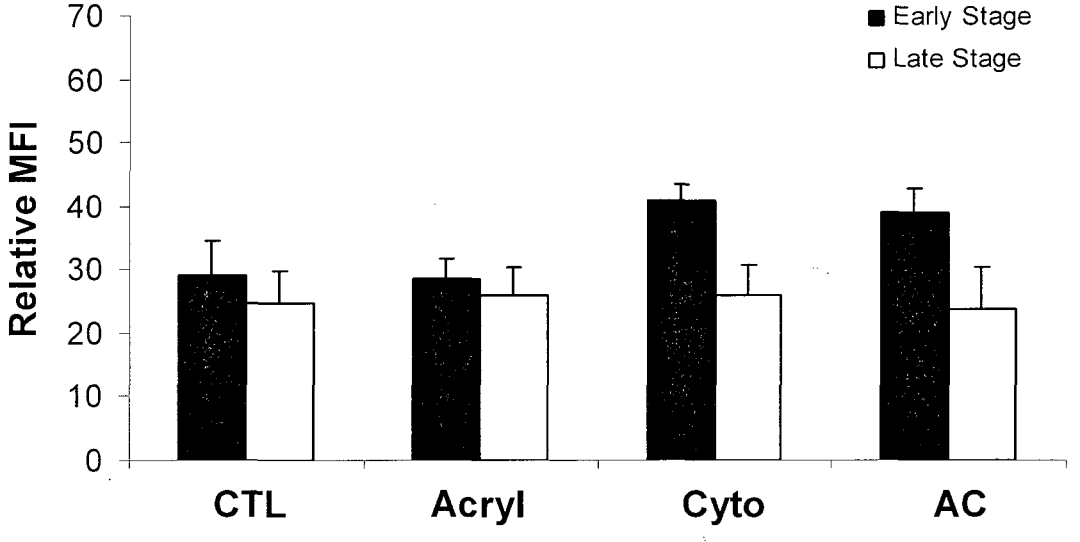


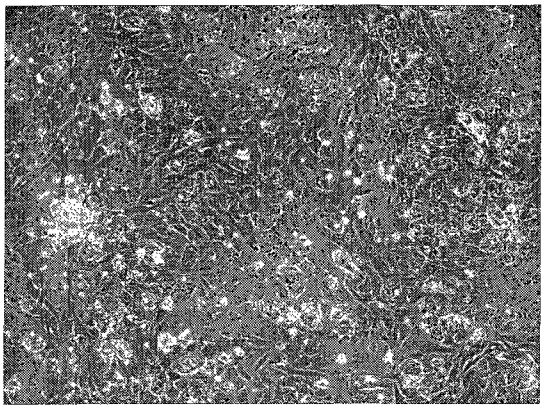
Figure 18b:



The effect of 48 hour cytokine treatment on the surface expression of Fas (mean ± SEM) of cultured luteal cells of early and late stage bovine CL (Fig. 18). Treatment did not alter the number of cells expressing Fas (Fig. 18a), or the relative mean fluorescence intensity within each stage (Fig. 18b). Different letters indicate significant differences ($P<0.05$; n=4 CL/stage). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines)

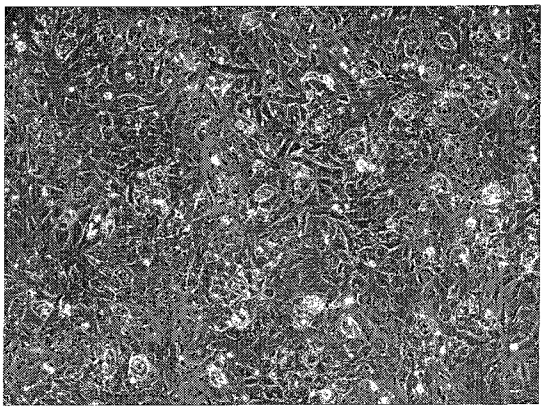
Figure 19: Early stage luteal cell death observed after 48 hours of cytokine treatment.

Figure 19a:



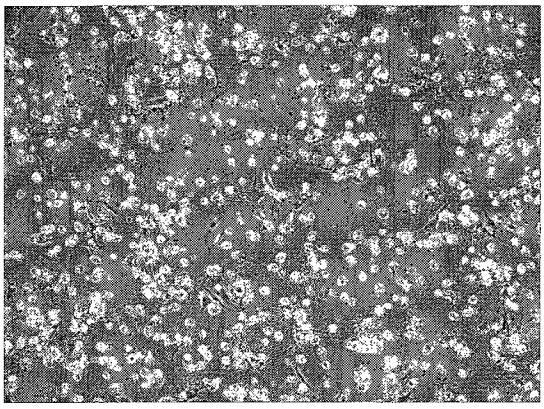
Control

Figure 19b:



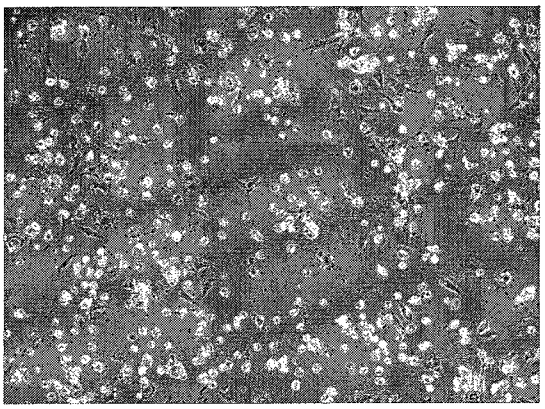
Acrylamide

Figure 19c:



Cytokines

Figure 19d:

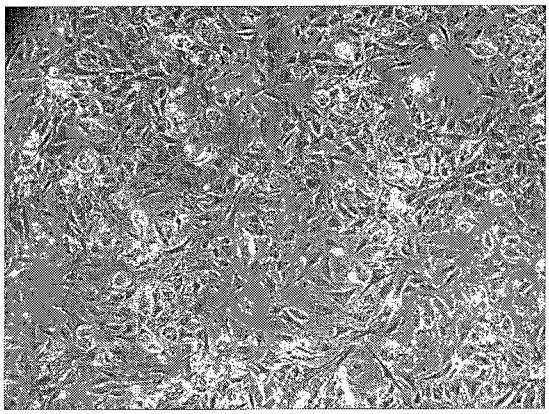


Acrylamide + Cytokines

Representative phase-contrast photomicrographs (20x magnification) of early stage bovine luteal cells following 48 hours of exposure to cytokines. Control (Fig. 19a) and acrylamide-treated (Fig. 19b) cultures were healthy, with a dense monolayer of mostly steroidogenic cells. Cytokine-treated (Fig. 19c,d) cultures contained substantial cell death as indicated by numerous phase-bright cells. Acrylamide pre-treatment did not enhance the effect of the cytokines (Fig. 19d).

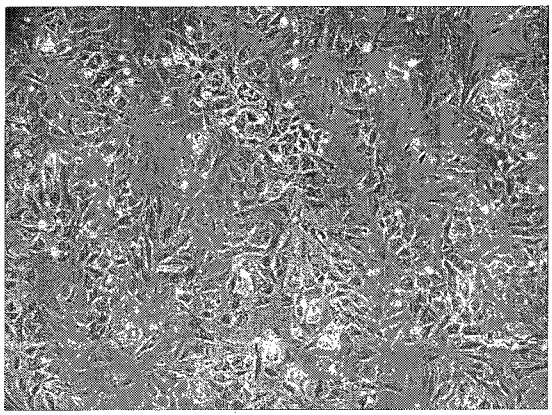
Figure 20: Late stage luteal cell death observed after 48 hours of cytokine treatment.

Figure 20a:



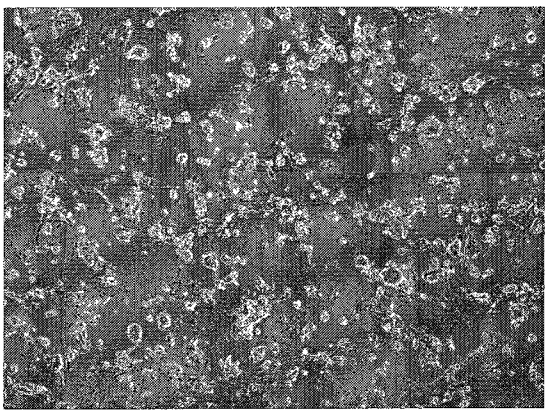
Control

Figure 20b:



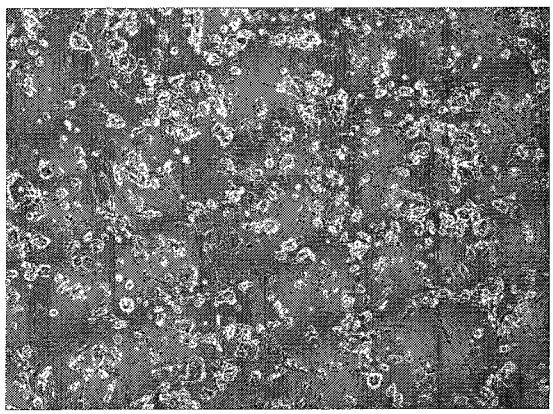
Acrylamide

Figure 20c:



Cytokines

Figure 20d:



Acrylamide + Cytokines

Representative phase-contrast photomicrographs (20x magnification) of late stage bovine luteal cells following 48 hours exposure to cytokines. Similar to early stage luteal cells, control (Fig. 20a) and acrylamide-treated (Fig. 20b) cultures were healthy, with a dense monolayer of mostly steroidogenic cells. Cytokine-treated (Fig. 20c,d) cultures contained substantial cell death as indicated by numerous floating cells. Acrylamide pre-treatment did not enhance the effect of the cytokines (Fig. 20d).

Figure 21: Quantitative assessment of cell death for cultured luteal cells from early and late stage bovine CL after 24 hours of cytokine treatment.

Figure 21a:

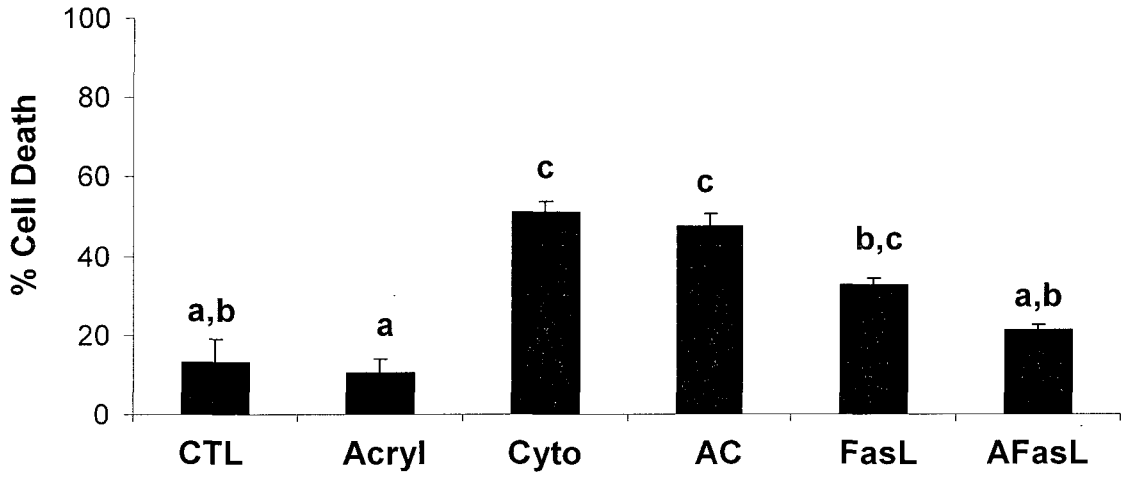
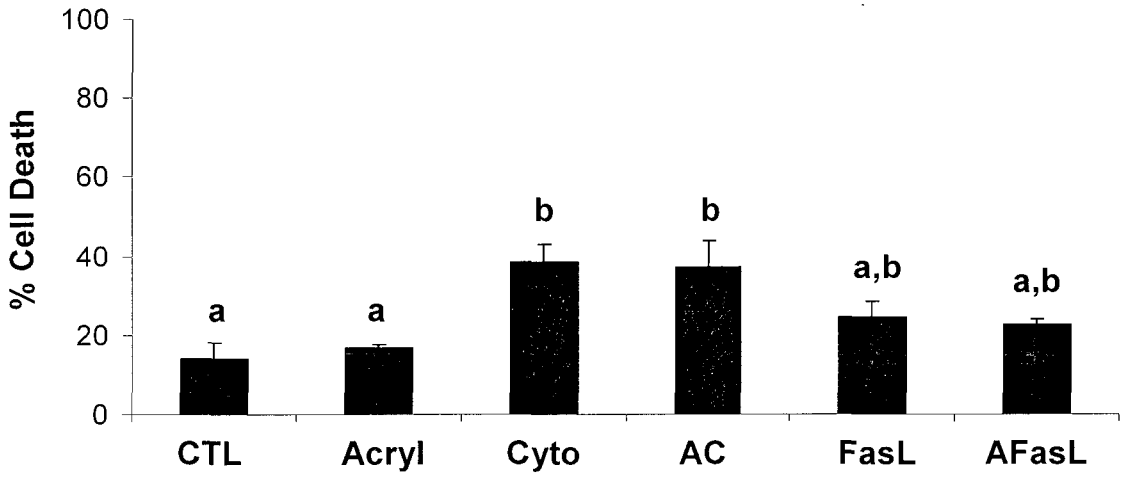


Figure 21b:



Cell death after 24 hour exposure to cytokines for luteal cells of early (Fig. 21a) and late stage bovine CL (Fig. 21; mean $\pm$ SEM). Cytokines induced cell death ($P<0.05$; n=4 CL/stage) in both early (Fig. 21a) and late (Fig. 21b) stage CL, whereas acrylamide had no effect ($P>0.05$). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines, FasL = Fas Ligand, AFasL = Acrylamide + Fas Ligand)

Figure 22: Quantitative assessment of cell death for cultured luteal cells from early and late stage bovine CL after 48 hours of cytokine treatment.

Figure 22a:

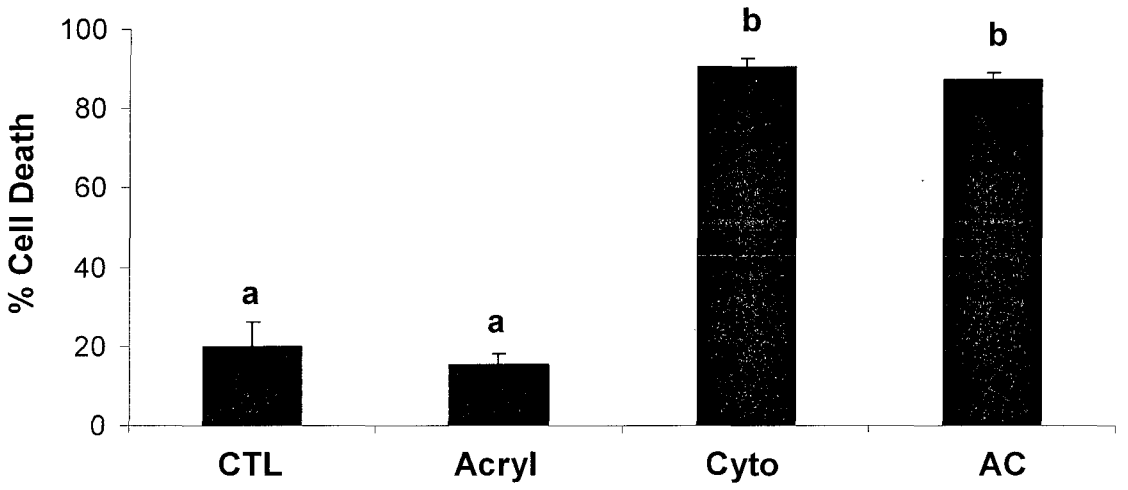
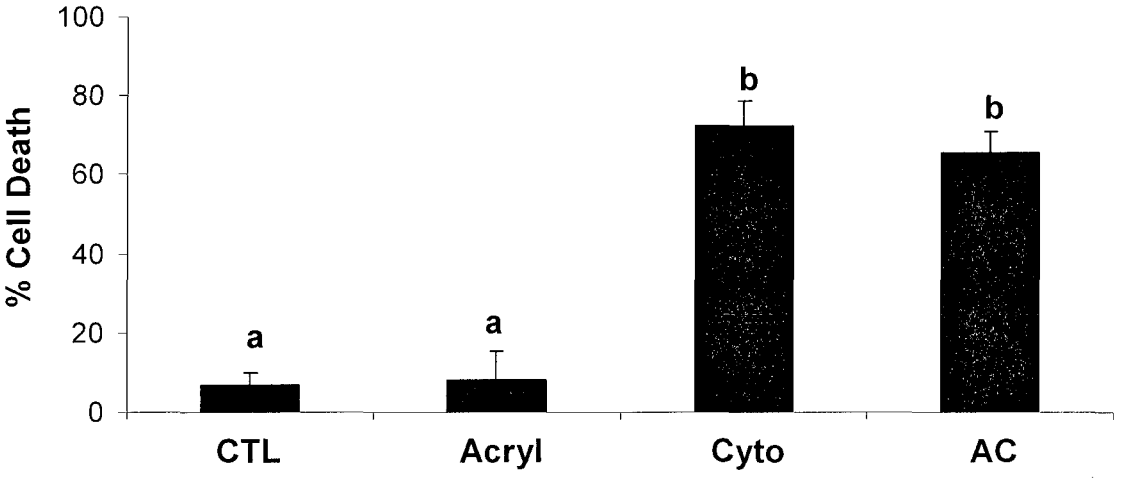


Figure 22b:



Cell death after 48 hour exposure to cytokines for luteal cells of early (Fig. 22a) and late stage bovine CL (Fig. 22b; mean $\pm$ SEM). Cytokines induced cell death ($P<0.05$; n=4 CL/stage) in both early (Fig. 22a) and late (Fig. 22b) stage CL, whereas acrylamide had no effect ($P>0.05$). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines)

Figure 23: Effect of cytokine treatment on cytokeratin-18 expression in bovine luteal cells as determined by flow cytometric analysis.

Figure 23a:

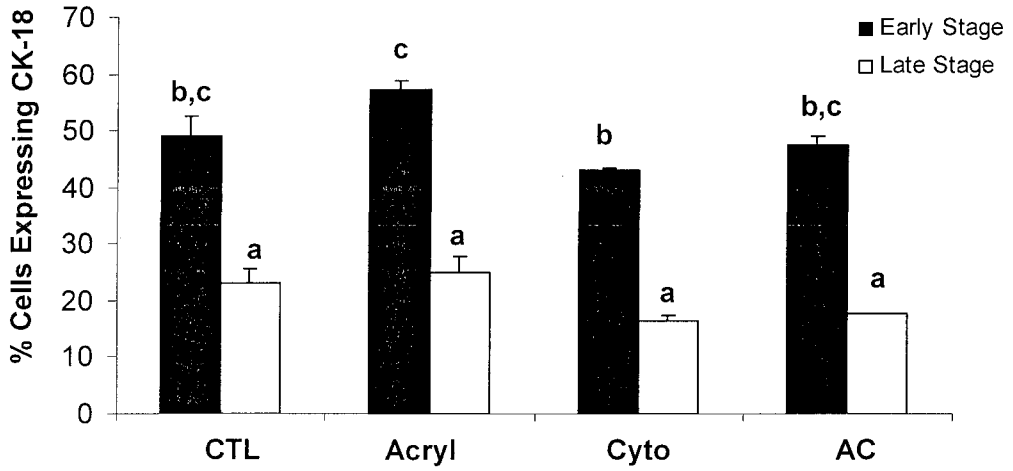
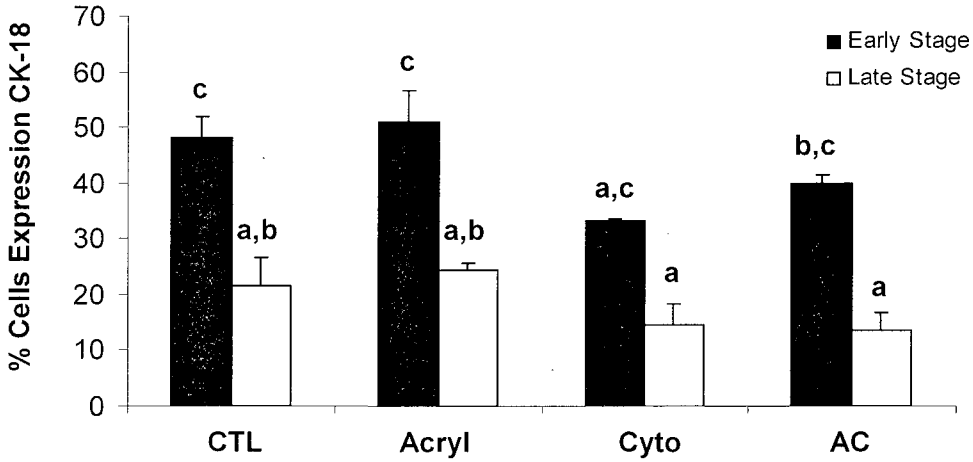


Figure 23b:



Cytokeratin-18 expression after 24 hours (Fig. 23a) and 48 hours (Fig. 23b) of cytokine treatment (mean ± SEM). Cytokines did not alter cytokeratin-18 expression for either stage. Acrylamide pre-treatment had no effect on cytokeratin-18 expression. Different letters indicate significant differences ($P<0.05$; n=2 CL/stage). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines)

Figure 24: Effect of cytokine treatment on progesterone secretion by cultured luteal cells of early and late stage bovine CL.

Figure 24a:

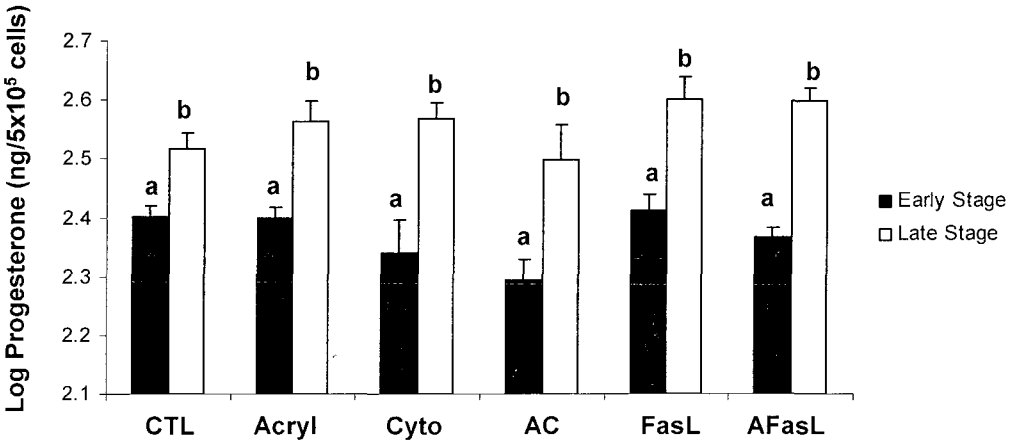
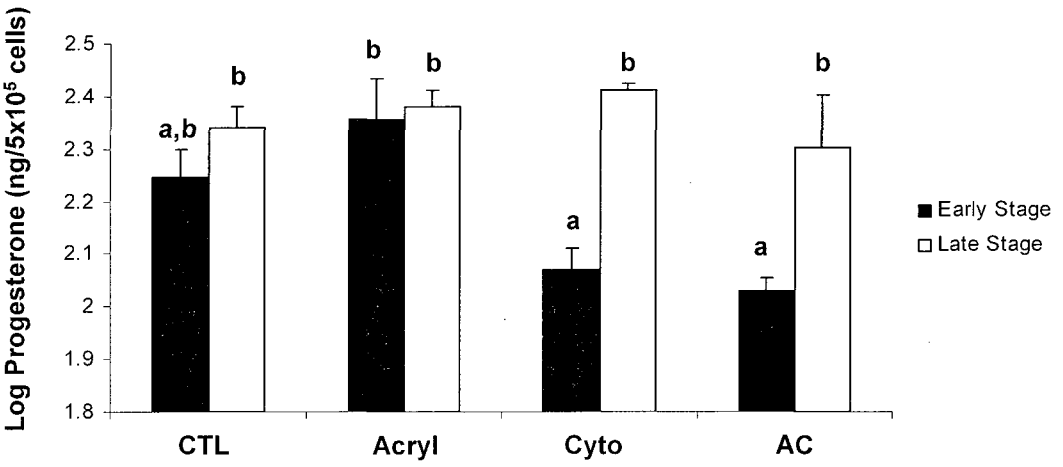


Figure 24b:



Progesterone secretion by cultured luteal cells of early and late stage bovine CL after 24 hour (Fig. 24a) and 48 hours (Fig. 24b) of cytokine treatment (mean $\pm$ SEM). After 24 hours cytokine treatment, progesterone secretion did not change. After 48 hours, progesterone secretion started to decrease in response to cytokine treatment. Pre-treatment with acrylamide did not alter progesterone secretion compared to controls. Different letters indicate significant differences ($P<0.05$; n=3-4 CL/stage). (CTL = control, Acryl = Acrylamide, Cyto = Cytokines, AC = Acrylamide + Cytokines, FasL = Fas Ligand, AFasL = Acrylamide + Fas Ligand)

Figure 25: Progesterone content in cellular fractions and conditioned medium of cultured bovine luteal cells following 24hr and 48hr exposure to acrylamide.

Figure 25a:

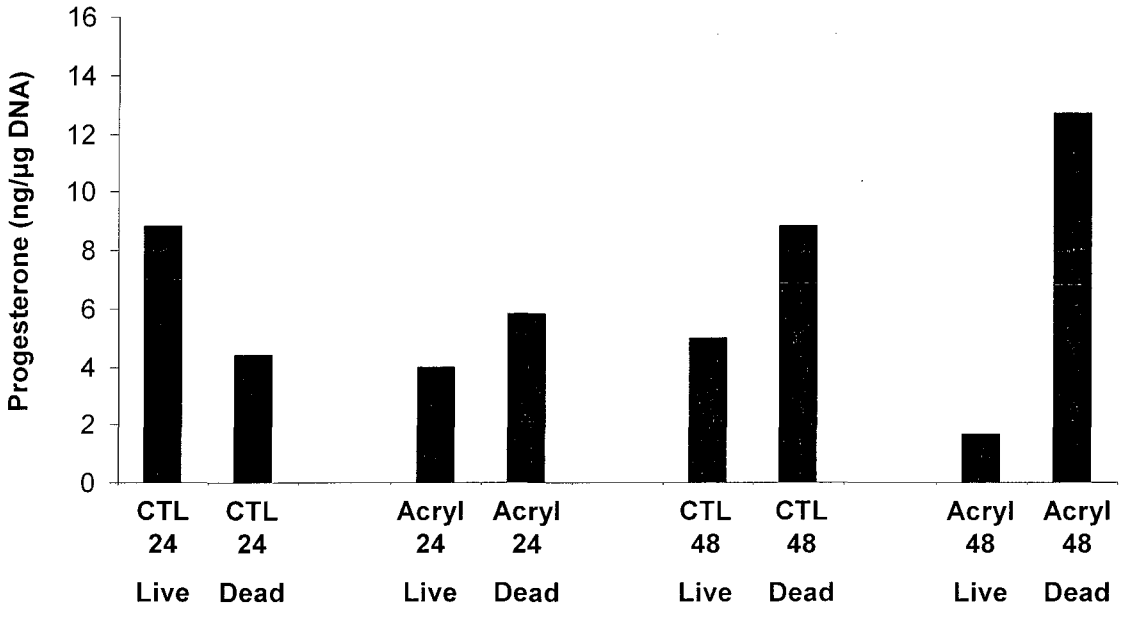
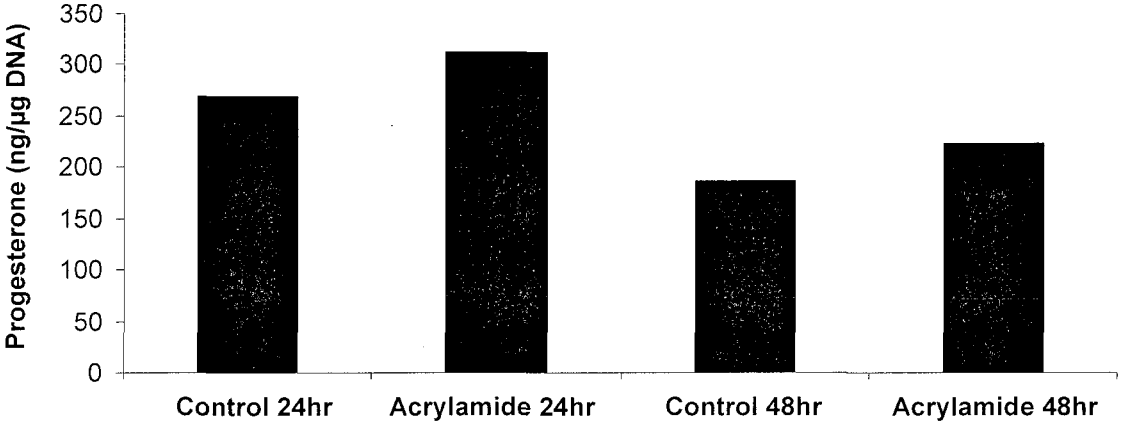


Figure 25b:



In 3 of 4 instances, the amount of progesterone extracted from dead cell fractions was higher than the corresponding live cell fractions (Fig. 25a) regardless of treatment or time of exposure. In acrylamide-treated cultures, progesterone secretion into the culture medium tended toward being higher than corresponding controls (Fig. 25b). (CTL = control, Acryl = acrylamide).

DISCUSSION

The present study evaluated the functional role of cytokeratin 18 (CK-18) filaments in FasL-induced apoptosis in bovine CL. We verified a previous report (135,137) that CK-18 filaments are differentially expressed in bovine CL. We also demonstrated for the first time that bovine luteal cells express Fas at the surface of the cell, with increased expression in early stage CL. Lastly, we determined disruption of CK-18 filaments with acrylamide does not enhance Fas expression at the surface of luteal cells, nor does it augment FasL-induced cell death.

Expression of CK-18 filaments is highest in early stage bovine CL and steadily decreases with maturity of the CL (135,137). The current study confirmed these observations while quantifying the extent of CK-18 expression by flow cytometry. We found that numbers of CK-18 positive cells decrease from early to late stage CL by 40%. Thus, it is tempting to consider a possible functional role for CK-18 filaments during the lifespan of the CL. Intermediate filaments provide mechanical integrity to cells and aid in the movement of lipid droplets within steroidogenic cells. Cells with intermediate filaments are more resistant to stress and strain than cells lacking these structures (118). There is also a close association between intermediate filaments and the movement of cholesterol during steroidogenesis, however it is still unknown how cholesterol moves in association with intermediate filaments (117). More recently, CK-18 intermediate filaments have been implicated in cellular resistance to apoptosis (85,127,129) and identified as targets of caspase cleavage during cell death (120,121). The mechanisms by which they provide resistance to cell death include sequestering Fas within the Golgi apparatus, preventing it from reaching the cell surface and interacting with FasL (85).

Additionally, CK-18 filaments sequester TRADD (TNF receptor associated death domain), preventing TNFα-induced cell death (129). Conversely, CK-18 filaments become targets of caspase cleavage when caspase-3 is activated during apoptosis (32,120,122). The cleavage of CK-18 leads to the collapse of the intermediate filament structure, which forms cytoplasmic inclusions in which CK-18 and caspase-3 are colocalized (123). It is these mechanisms of resistance and susceptibility that formed the basis for investigating the potential role of CK-18 intermediate filaments in the current study.

The observation of quantifiably higher Fas receptor expression at the surface of early stage than late stage luteal cells was unexpected and at odds with previous studies. In previous work, investigators examined Fas mRNA (69, 102) and protein expression (91,93) in mice and rat ovaries, and they found that Fas increased in CL undergoing regression. Much of their conclusions, however, were drawn from RT-PCR (69,102) and immunohistochemical data (91,93). Here, Fas was quantified using dissociated bovine luteal cells and flow cytometry. We would argue that immunohistochemical staining is generally not considered a quantitative approach to measure protein expression, although it would permit the detection of Fas expression in a wide range of cell types compared to the current study. Typical ovarian tissue sections contain steroidogenic cells, fibroblasts, endothelial cells, immune cells, and red blood cells that are all assessed collectively for Fas expression. Dissociation of luteal tissue, as described in the current investigation, removes many of these cell types, creating a relatively pure suspension of steroidogenic cells with a few contaminating endothelial cells and fibroblasts. Thus, a shortcoming of the present study is that not all cell types within the CL were assessed for Fas expression.

Moreover, species-specific differences in Fas expression might account for the discrepancy in temporal expression of Fas throughout the luteal phase between the current study and the cited previous studies. Overall, we observed a 72% decrease in the number of luteal cells expressing Fas, and a 59% decrease in the density of Fas expressed at the cell surface across the luteal phase. Total Fas expression (surface and intracellular) for freshly isolated cells was higher for early stage than late stage CL. For cultured luteal cells, Fas surface expression followed a similar pattern, but was enhanced by culture particularly in late stage cells. Exposure of the cultured cells to FasL, however, resulted in similar numbers of cell death for both stages of the luteal phase. This indicates cells from both stages of CL are equally susceptible to FasL-mediated cell death despite differences in the cell surface expression of Fas.

The observation that Fas expression is elevated on luteal cells of early stage CL without enhancing their susceptibility to FasL-induced death indicates mechanisms within the cell guard against FasL-induced apoptosis. In other types of cells, a soluble, secreted isoform of Fas has been identified that sequesters FasL prior to binding at the cell surface, thus preventing cell death (141-143). This isoform of Fas lacks the transmembrane domain of wild type Fas, causing it to be secreted rather than expressed on the surface of cells (141). Soluble Fas is biologically active, retaining its ability to bind FasL, which interferes with the induction of apoptotic signaling by interacting with FasL before it is able to reach active Fas receptors on the surface of cells (141-143). More recently, Komatsu et al. demonstrated the expression of soluble Fas mRNA in cultured murine luteal cells, which decreases following treatment with the apoptotic cytokines, TNFα and IFNγ (144). Thus, it is possible a soluble form of Fas exists within

bovine luteal cells to provide protection, but this mechanism does not account for the overall high expression of Fas observed on the surface of luteal cells of early stage CL in the current study.

The phenomenon of membrane-bound splice variants of cytokine receptors might account for our observations of elevated Fas surface expression. The cytokine TRAIL (TNF-related apoptosis-inducing ligand) is structurally similar to FasL, and its receptors, DR4 and DR5, belong to the TNF receptor superfamily. After the initial discovery of a soluble, protective isoform of Fas, membrane-bound decoy receptors were also discovered for the cytokine TRAIL. These receptors, DcR1 and DcR2, have a cytoplasmic domain structurally similar to DR4 and DR5, respectively, but lack the intracellular death domain necessary for transmitting an apoptotic signal (145-147). The TRAIL decoy receptors are expressed in several tissues, but within the ovary, only DcR2 is observed (145-147). The existence of decoy receptors to cytokines structurally similar to FasL makes plausible the idea that decoy receptors for FasL also exist. Recently, Jenkins et al. (2000) discovered a membrane-bound Fas decoy receptor in thymocytes. Similar to DcR1 and DcR2, the Fas decoy receptor contains an extracellular and cytoplasmic domain similar to wild type Fas, but lacks the intracellular death domain. Unlike soluble Fas, the decoy receptor is expressed on the plasma membrane and retains its ability to bind FasL, but does not induce cell death (148). These observations tempt us to consider that a decoy receptor of Fas might exist on bovine luteal cells, explaining the unexpected, high expression of Fas on surface of cells during early CL development. However, further characterization of the Fas receptor on bovine luteal cells would need to

be conducted to determine whether or not a Fas decoy receptor does in fact exist within the bovine ovary.

A second alternative explanation for the high, unanticipated expression of Fas in early stage CL compared to late stage CL might relate to systemic progesterone concentrations. Recall that plasma progesterone concentrations for cows providing the late stage CL were higher than those providing early stage CL. High systemic concentrations of progesterone might directly impair Fas surface expression and/or actions in bovine luteal cells. In the rat, for instance, high progesterone concentrations down-regulate Fas mRNA expression (149). In cultured bovine luteal cells, the progesterone antagonist onapristone increases Fas mRNA expression (17), suggesting that indeed progesterone directly impairs Fas expression on luteal cells. Further experimentation will be required, however, to critically examine this idea.

Acrylamide was utilized in the present study to disrupt cytokeratin intermediate filaments. Acrylamide selectively disrupts intermediate filaments without otherwise adversely affecting the cells (150-155). For instance, in fibroblasts (150) and epithelial cells (151), exposure to acrylamide causes the filaments to form juxtanuclear aggregates, yet microtubules are unaffected. Others have shown the filaments do not become fully disassembled (152), but rather undergo acute dephosphorylation. Dephosphorylation provokes a 50% loss of phosphate from the keratin protein which corresponds with the morphological changes in intermediate filament expression (156). However, the dephosphorylation event is transient, and the striking changes in intermediate filament organization are reversible. Filaments re-establish their 'net-like' organization within 12 hours after acrylamide removal (151), and complete rephosphorylation occurs within 18

hours (156). Although acrylamide generally targets intermediate filaments, some investigations have discovered some other non-specific effects. Arocena (2006), for example, showed that acrylamide disrupts intermediate filaments, microfilaments, and microtubules in bovine lens epithelial cells. Shiver et al. (1992) determined acrylamide promotes steroidogenesis in murine adrenal cells (158). In the Shiver (1992) study, adrenal cells underwent a change in morphology, becoming rounded. This change in morphology corresponded with the acrylamide-induced increase in steroid production (158), leading the authors to conclude that acrylamide influences steroid production by enhancing the movement of cholesterol ester-rich lipid droplets and/or the delivery of cholesterol to the mitochondria (158). There is a close association between intermediate filaments and lipid droplets (117), and disruption of the filaments is thought to liberate the droplets and allow them to move more freely within the cell (158). Additionally, the change in cell shape and the disruption of intermediate filament-mitochondrial interactions may facilitate the movement of the mitochondria, in essence enhancing the delivery of the lipid droplets to the mitochondria (158). In the present study, acrylamide targeted CK-18 intermediate filaments without affecting the integrity of other cytoskeletal filaments (e.g. microtubules). Additionally, acrylamide did not alter cellular morphology or steroidogenic production by the bovine luteal cells (Fig. 12). From this we conclude that acrylamide disrupts CK-18 filaments in bovine luteal cells without affecting normal cellular processes.

The concept that CK-18 filaments regulate Fas trafficking was first proposed in a study in which murine hepatocytes were evaluated (85). Using a knock-out mouse model, the authors discovered CK-18 filaments mediate Fas mobilization from the Golgi

apparatus to the plasma membrane (85). Mouse genetic knockouts have proven useful as tools for permanently and completely eliminating a protein of interest. In the current study, the chemical acrylamide was used in an attempt to mimic the effects of intermediate filament loss seen in genetic knockout studies. An important difference between previous knock-out studies (85,159) and the current study is that acrylamide provides only transient disruption of the filaments (151,156). These transient actions of acrylamide might account for the lack of detectable change in Fas expression on the surface of cells from early and late stage CL. A suggestion would be to identify a cytokeratin-disrupting agent that has specific, long-term effects and then re-assess the surface expression of Fas on bovine luteal cells in this context.

The susceptibility of bovine luteal cells to FasL-induced apoptosis after CK-18 disruption was assessed in the current study by exposing the cells to a cytokine cocktail containing FasL. The rationale for the use of a cytokine cocktail is based upon the knowledge that in bovine CL, T lymphocytes and macrophages increase before the onset of luteolysis (47-49). Hence, as these immune cells invade the CL, it is presumed they release a cocktail of cytokines (i.e., TNFα, IFNγ, FasL), which collectively contribute to luteal cell apoptosis and the regression of the CL (67,69,72). In fact, most previous studies utilize a combination of cytokines such as TNFα , IFNγ , and FasL, arguing that they enhance Fas mRNA (69,96,101,109,110) and protein expression (74,104,110). Notably, the cytotoxic effects of FasL become evident only when combined with TNFα and IFNγ (69,89,90,95). For these reasons, a similar cytokine cocktail containing TNFα, IFNγ, and FasL was utilized in the current series of experiments to induce cell death in the luteal cell cultures.

Another manner in which cytokines contribute to the regression of the CL is by inhibiting gonadotropin-stimulated progesterone production (67,72). In mid-cycle bovine luteal cultures exposed to luteinizing hormone (LH), TNFα (67) and IFNγ (72) prevented the increase in progesterone secretion that was observed in cultures treated with LH alone. In the current study, we observed a decrease in progesterone production by luteal cells of early stage CL after 48 hrs of cytokine treatment, yet no change in progesterone secretion was observed for luteal cells of late stage CL. The lack of change in progesterone secretion by late stage luteal cells was unexpected given the previous observations that TNFα decreases progesterone concentrations in late stage luteal tissue (54). In the present study, progesterone in extracts of live vs. dead cell pellets, as well as the secreted progesterone in the culture medium, was measured to determine the influence, if any, that dying cells have on progesterone production. No striking differences were observed in the amount of progesterone secreted into conditioned medium of control and death-induced cultures. However, surprisingly, extracts of dead cell pellets tended to contain more progesterone than their live cell counterparts, especially after 48hrs of acrylamide exposure. Perhaps the components of dead cell fractions sequester steroids? Nevertheless, the overall results of this single experiment indicate that in late stage luteal cells, steroidogenesis is maintained and/or metabolism decreased to sustain a constant amount of progresterone in the conditioned medium following cytokine exposure. Similar effects are not observed for luteal cells of early stage CL.

The inability of CK-18 filament disruption (via acrylamide) to enhance Fas expression in the current experiments was disappointing. Was the lack of effect due to

the short-term, transient actions of acrylamide? To address this concern, we evaluated the hepatocellular carcinoma cell line, HepG2, as a "positive control" cell type to test our proof of concept. Recall hepatocytes were the cell type of choice for demonstrating CK-18 disruption and Fas regulation in the mouse genetic knock-out studies (85). As anticipated, exposure of HepG2 cultures to acrylamide caused peri-nuclear aggregation of CK-18 filaments and enhanced the number of cells expressing Fas on the cell surface. These observations validated that indeed acrylamide-induced disruption of CK-18 filaments enhances the cell surface expression of Fas. However, in HepG2 cells, we also observed that acrylamide disrupted microtubule organization. It is possible that both CK-18 filaments and microtubules, particularly in hepatocytes, influence the cell surface expression of Fas. It is also conceivable that microtubules influence Fas expression in luteal cells. In at least one study, disruption of microtubules increased Fas mRNA expression but, surprisingly, triggered a decrease in Fas protein expression at the cell surface (160). Other studies show that anti-cancer drugs disrupt microtubules, which enhance FasL expression and the incidence of apoptosis (161). Given the current results and the potential relationship between microtubule disruption and FasL, it merits further study to examine the role of microtubules in Fas trafficking in bovine luteal cells.

The current study evaluated the influence of CK-18 filaments on Fas trafficking and FasL-induced death of bovine luteal cells. At present, the results do not support the hypothesis that CK-18 intermediate filaments regulate the movement of Fas to the surface of the cell. We observed that acrylamide disrupts CK-18 filaments without enhancing Fas surface expression or FasL-induced cell death. Noting that the effects of acrylamide are transient and reversible, it is conceivable that CK-18 filaments do play a role in

apoptosis and regression of the bovine CL, but acrylamide is not the agent of choice to elucidate that role. Collectively, we conclude that if CK-18 filaments regulate Fas expression and FasL-induced death in bovine luteal cells, their effects are not as profound as has been observed in other, non-ovarian cell types such as hepatocytes.

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APPENDIX

Institutional Animal Care and Use Committee Approval

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Project: Role of Cytokeratin 8/18 Filaments in Fas Ligand-Induces Apoptosis of Ovarian Steroidogenic Cells: Mechanisms of Gonadotopin Action and Oxidative Stress During Folliculogenisis and Atresia
Category: C
Approval Date: 25-Feb-2009

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.* The IACUC made the following comment(s) on this protocol:

1. In Section IV, A (experimental design), the IACUC added at the end of the penultimate sentence the following statement, "...using a 20 gauge needle."

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

Corpus Luteum Dissociation and Cell Culture

Day Before Dissociation:

AUTOCLAVE – 30 minutes, with 10 minute dry time

- cutting board
- 2 scissors
- forceps (fine-tip and blunt tip)
- hemostat
- 25mL Erlenmeyer flask
- 30mL beaker
- 2-10mL beakers
- ½ glass Petri dish, cover slide
- spinner flask (not in envelope)
- zippet
- razor blade

Day of Surgery (Day -1):

PREPARATION

-Make Ham's F-12 + gentamicin

-Add 200μL gentamicin to 100mL Ham's F-12 sterile media.

-Place in 2 sterile specimen containers. Place on ice.

-Swipe hood with Poviodine.

-Set up water bath for 37°C (Temperature control on 2 to 2 ¼). → too cold, collagenase doesn't work, too hot, it kills cells

-Obtain surgery bucket.

-Make BSA Media (0.5g / 100mL med + 200 μL gent)

BLOOD SAMPLE

-Spin blood for 20 minutes at 3000 rpm, 4°C. Remove plasma to dram vial and freeze.

FLASK PREPARATION (do this during tissue preparation or the day before luteectomy)

-Thaw one tube of FBS in water bath.

-Add 10mL calf serum to 100mL Ham's F-12 with 300μL gentamicin.

-Add 2.5mL to each flask to be used. Swirl to cover bottom.

-Add 500μL to each chamber of an 8-chamber slide

-Incubate at least 1 hour (up to 5 hours is okay).

-Rinse flasks with 2.5mL Ham's F-12 w/gentamicin right before use.

-Dump this 2.5mL out and add another 2.5mL of Ham's w/gentamicin

TISSUE PREPARATION

-Determine weight of Corpus Luteum (keep in media).

-cut off connective tissue (grey), place CL back in cup, take to scale, place CL on weigh boat and weigh

-Set up sterile equipment on cutting board (in hood).

-Weigh out collagenase for later use:

$$\frac{(2000)(\text{wt. of CL in grams})}{*\text{Activity of collagenase}} = \text{mg collagenase to use}$$

*collagenase activity is stated on bottle and will vary with each batch

Units = U/mg (210 U/mg)

-Place some BSA medium in a 10mL beaker. Take ¼ or ½ of CL tissue and Slice into 1mm strips. Hold onto tissue with rough edge of cover slide. Place slices into beaker containing BSA media.

-Finish with rest of CL.

-Using scissors, cut slices within the beaker into 1-2mm cubes (usu. 5-10 minutes).

-Pour cubed pieces into spinner flask. Rinse beaker 2X with BSA media and place media in spinner flask.

-Place spinner flask in water bath and spin on medium-low (2-3) for 1 minute. Let tissue settle and pour liquid out side (this contains excess red blood cells and other debris).

DISASSOCIATION

****make sure waterbath is at 37C before adding spinner flask containing the cells****

-Add collagenase directly to the spinner flask and bring the total volume up to 40mL with Ham's F-12 + Gent + BSA

-Spin for 1 hour.

-Every 10 minutes, aspriate contents of spinner flask with a wide mouth pipette (i.e., a broken-tip pipette).

AFTER 1 HOUR DISASSOCIATION – RINSING AND CENTRIFUGATION

1) Pour off supernatant of spinner flask into one 50-mL centrifuge tube (vent one end of the spinner flask and pull cap off the other end) - - with smaller CL, use 15mL tube
2) Spin at 228xg, 4°C for 10 minutes.
3) Pour off supernatant (asap since collagenase is still present), then bring volume up to 50mL with Ham's F-12 w/gentamicin. Use Pasteur pipette (plugged and autoclaved) to break up pellet (squeeze up and down). - - with smaller CL, use 15mL tube and bring total volume up to 15mL
4) Spin at 129xg, 4°C for 10 minutes.
5) Repeat step #3.

6) Spin at 57xg, 4°C for 10 minutes.
7) Repeat steps #3 and #6. (if supernatant isn't clear)
8) Pour off supernatant from centrifuge tubes. Bring up to 5mL with Ham's F-12
9) w/gentamicin.
10) Aspirate with Pasteur pipette to break up pellet.
11) Place cells on ice.

SECOND DISASSOCIATION

-Add collagenase directly to spinner flask and bring up to 40mL with Ham's + Gent + BSA

(smaller CL in smaller spinner flask, bring up to 20mL)

AFTER SECOND DISASSOCIATION – RINSING AND CENTRIFUGATION

1) Pour off supernatant on spinner flask into one 50-mL centrifuge tube (vent one end of the spinner flask and pull cap off the other end)
2) Spin at 1000 rpm, 4°C for 10 minutes.
3) Pour off supernatant, then bring volume up to 50mL with Ham's F-12 w/gentamicin. Use Pasteur pipette (plugged and autoclaved) to break up pellet (squeeze up and down).
4) Spin at 129xg, 4°C for 10 minutes.
5) Repeat step #3.
6) Spin at 57xg, 4°C for 10 minutes.
7) Repeat steps #3 and #6. (if supernatant isn't clear)
8) Pour off supernatant from centrifuge tubes. Bring both up to 5mL with Ham's F-12
   w/gentamicin.
9) Apirate with Pasteur pipette to break up pellet.
10) Combine tubes and bring total volume up to 20mL with Ham's F-12 w/gentamicin
11) Bring total volume up to 50mL and spin at 500rpm, 4C, 10 minutes
12) Resuspend pellet in 20mL (10mL for smaller CL)

CELL COUNTING BY TRYPAN BLUE EXCLUSION METHOD

- Dilute cells – dilution depends on what the pellet looks like. Dilution range is 1:5 to 1:20.
- Usually do a 1:10 (1:5 for a small pellet).
- Dilution of cells: 1:5 = 200µL cells + 800µL trypan blue dilution (see below*)
- 1:10 = 100µL cells + 900µL trypan blue dilution
- Trypan blue dilution*: Figure out amount of trypan blue needed for cell dilution X 2 for 2 tubes. Make a round mL amount higher than necessary. Ex: if 3mL of trypan blue dilution will be sufficient, use 2.7mL sterile PBS and 300µL trypan blue (or use working solution 1:10).

- -To dilute cells: Place required amount of trypan blue dilution in each of 2 – 12x75 assay tubes (BE CAREFUL – assay tubes are not sterile). Add amount of cells indicated for your 1:5 to 1:20 dilution of cells.
- -Vortex quickly, let sit 30 seconds to 1 minute.
- -Vortex quickly, then load both sides of hemocytometer: Place a drop of solution on each side (use one drop from each tube). Blot excess once drop has seeped in.
- -Microscope: Find cells at 10X, count at 40X. Use fours corners plus middle region of hemocytometer for counting.

- Calculate average # of cells/square. If counts from each side are very different, do another dilution.
- Calculate average # of cells in solution:
  1:10 dilution –> (total # of cells)*(10^4)*(dilution factor {10, for a 1:10}) = # cells/mL
  Ex. 84.5 average cells/square = $8.4x10^6$ or 84.5 million cells/mL
  Divide by 2 for a 1:5 dilution.

Freshly Isolated Cell Fixation Protocol

1. Aliquot 300μL ($1.5x10^6$ cells) of cells into screen-capped centrifuge tubes
2. Spin at 176xg, 10 minutes, 4°C
3. Resuspend pellet with 100μL Ham's F12 and 400μL 2% paraformaldehyde in 1xPBS
   (therefore each tube contains $1.5x10^6$ cells/tube/800μL 1% paraformaldehyde)
4. Place tubes on ice for 2 hours, flicking occasionally
   a. If not permeabilizing cells, after 2 hours of fixation, place tubes in 4°C fridge. If permeabilizing cells, follow rest of protocol
5. Spin all tubes at 176xg, 5min, 4°C
6. Discard supernatant by pouring and dabbing
7. Add 4mL 1xPBS to all tubes
8. Spin at 176xg, 5min, 4°C
9. Add 4mL 1xPBS to all tubes
10. Spin at 176xg, 5min, 4°C
11. Discard supernatant by pouring and dabbing
12. Resuspend pellet in residual 1xPBS and add 900μL 70% chilled ethanol
13. Place tubes in -20°C freezer until flow cytometric analysis

Fixation of Chamber Slides Protocol

1. Rinse slides 2x with 1xPBS
2. Fix cells with 2% paraformaldehde in 1xPBS for 20 minutes at room temperature
3. Rinse slides 2x with 1xPBS
4. Store slides in 1xPBS at 4°C until immunocytochemistry

Fixation of Flasks (T25) Protocol

1. Remove media
2. Rinse cells 1x with 3mL HBSS
3. Add 0.5mL trypsin-EDTA to each flask
4. Rock flasks gently; remove trypsin
5. Repeat steps 3 and 4
6. Let flasks stand for 10 minutes
7. Add 4mL Ham's F12 + 10% FBS to each flask – pipette up and down to remove all cells
8. Place 4mL of cell suspension into non-screen capped tubes
9. Spin tubes at 276xg, minutes, 4°C
10. Remove supernatant and resuspend cell pellet with 200μL Ham's F12
11. Spin tubes at 276xg, 5 minutes, 4°C
12. Add 200μL Ham's F12 and 400μL 2% paraformaldehyde in 1xPBS to all tubes (therefore each tube contains 800μL 1% paraformaldehyde)
13. Place tubes on ice for 2 hours, flicking occasionally
    a. If not permeabilizing cells, after 2 hour fixation, place tubes in 4°C fridge. If permeabilizing cells, follow rest of the protocol
14. Spin tubes at 276xg, 5 minutes, 4°C
15. Remove supernatant
16. Resuspend cell pellet in 4mL 1xPBS
17. Spin tubes at 276xg, 5 minutes, 4°C
18. Repeat steps 15-17
19. Remove supernatant and resuspend cell pellet in 900μL chilled 70% ethanol. Place tubes in -20°C freezer until flow cytometric analysis

Saving Conditioned Media and Floating Cells Protocol

1. Remove conditioned media from flasks (step 1 from Fixation of Flasks Protocol)
2. Place conditioned media in non-screen capped tubes
3. Spin tubes at 276xg, 5min, 4oC
4. Save 1mL supernatant, place in microcentrifuge tubes and place in -20oC freezer
5. Discard remaining supernatant
6. Resuspend cells in 400μL Ham's F12 and 400μL 2% paraformaldehyde in 1x PBS for 2 hours, on ice.
7. At the end of the fixation, add fixed cells to trypsinized cells from the flasks

Immunocytochemistry Protocol

1. Wash slides 2x with PBS with 0.1% BSA
2. Perform a blocking and permeabilization step: Incubate slides with 200μL of 1% BSA + 10% NGS + 0.3% triton x-100 in 1xPBS for 1hr at room temperature
3. Wash slides 2x with PBS with 0.1% BSA
4. Add primary antibody
    a. CK18 non-conjugated primary: 1:800 dilution with PBS with 1.0% BSA + 10% Normal Goat Serum (NGS) + 0.3% triton x-100
    b. Microtubule (MT) primary: 1:200 dilution with PBS with 1.0% BSA + 10% NGS
    c. Neg CTL: PBS with 1.0% BSA + 0.3% triton x-100
5. Incubate slides overnight at 4°C in a humidified chamber
6. The next day, wash slides 3x with PBS with 0.1% BSA
7. Add secondary antibody
    a. Alexa 488 secondary (for CK18): 1:200 dilution with PBS with 1.0% BSA + 10% NGS + 0.3% triton x-100
    b. Texas red secondary (for MT): 1:200 dilution with PBS with 1.0% BSA + 10% NGS + 0.3% triton x-100
8. Incubate slides at 37°C for 1hr in a humidified chamber
9. Wash slides 2x with PBS with 0.1% BSA
10. Counterstain and mount with DAPI mounting medium

Flow Cytometry Protocol: Fas Staining

1. Spin all tubes at 276xg, 5 min, 4°C
2. Remove supernatant by pouring and dabbing
3. Resuspend pellet in 1mL PBS with 0.1% BSA
4. Spin all tubes at 276xg, 5 min, 4°C
5. Remove supernatant by pouring and dabbing
6. Resuspend pellet in 1mL PBS with 0.1% BSA
7. Spin all tubes at 276xg, 5 min, 4°C
8. Aspirate supernatant with Pasteur pipette
9. Add 100μL primary antibody or PBS with 1.0% BSA (to neg CTLs)
    a. Fas primary antibody: 20μg of antibody in PBS with 1.0% BSA + 10% NGS
10. Incubate tubes overnight at 4°C wrapped with parafilm
11. Add 1mL PBS with 0.1% BSA to all tubes
12. Spin tubes at 276xg, 5 min, 4°C
13. Remove supernatant by pouring and dabbing
14. Add 1mL PBS with 0.1% BSA to all tubes
15. Spin tubes at 1100rpm, 5 min, 4°C
16. Aspirate supernatant with Pasteur pipette
17. Add 100μL secondary antibody or PBS with 1.0% BSA (to neg CTLs)
    a. Alexa 488 secondary antibody: 1:200 with PBS with 1.0% BSA + 10% NGS
18. Incubate tubes for 1hr at 37°C wrapped with parafilm
19. Add 1mL PBS with 0.1% BSA to all tubes
20. Spin tubes at 276xg, 5 min, 4°C
21. Remove supernatant by pouring and dabbing
22. Add 1mL PBS with 0.1% BSA to all tubes
23. Spin tubes at 276xg, 5min, 4°C
24. Remove supernatant by pouring and dabbing and resuspend in 0.5mL 1xPBS

Flow Cytometry Protocol: Cytokeratin Staining

1. Spin all tubes at 276xg, 5 min, 4°C
2. Remove supernatant by pouring and dabbing
3. Resuspend pellet in 1mL PBS with 0.1% BSA
4. Spin all tubes at 276xg, 5 min, 4°C
5. Remove supernatant by pouring and dabbing
6. Resuspend pellet in 1mL PBS with 0.1% BSA
7. Spin all tubes at 276xg, 5 min, 4°C
8. Aspirate supernatant with Pasteur pipette
9. Add 100µL primary antibody or PBS with 1.0% BSA (to neg CTLs)
    a. CK FITC-conjugated primary antibody: 1:100 dilution with PBS with 1.0% BSA
10. Incubate tubes for 1hr at 37°C with a parafilm wrap
11. Add 1mL PBS with 0.1% BSA to all tubes
12. Spin tubes at 276xg, 5 min, 4°C
13. Remove supernatant by pouring and dabbing
14. Add 1mL PBS with 0.1% BSA to all tubes
15. Spin tubes at 276xg, 5 min, 4°C
16. Remove supernatant by pouring and dabbing and resuspend in 0.5mL 1xPBS

Cell Fraction Protocol (for progesterone measurement)

Conditioned Media and Dead Cell Pellet

1. Remove conditioned media from flasks and place in non-screen capped tubes
2. Spin tubes at 276xg, 5 min, 4°C
3. Save 1mL conditioned media (supernatant), place in microcentrifuge tubes, and place in -20°C freezer
4. Remove remaining supernatant and resuspend cell pellet ("dead cell pellet") in 100µL 1xPBS – transfer cell pellet in 1xPBS to microcentrifuge tube and place in -20°C freezer

Live Cell Pellet

5. Rinse each flask with 3mL HBSS
6. Remove HBSS
7. Add 0.5mL trypsin-EDTA to each flask
8. Rock gently; remove trypsin-EDTA
9. Add 0.5mL trypsin-EDTA to each flask
10. Rock gently; remove
11. Let flasks stand for 10 minutes
12. Add 4mL Ham's F12 + 10% FBS to each flask – pipette up and down to remove all cells
13. Add 4mL cell suspension to non-screen capped tubes
14. Spin tubes at 276xg, 5 min, 4°C
15. Remove all supernatant by pouring and dabbing
16. Add 100µL 1xPBS to cell pellet ("live cell pellet")
17. Transfer cell pellet in 1xPBS to microcentrifuge tube and place in -20°C freezer

DNA Extraction and DNA Assay

TNE Buffer – pH 7.4
Tris 10mM
EDTA 1mM
NaCl 100mM

1. Measure out 50% of final volume of MilliQ water
2. Add individual ingredients and stir to completely dissolve ingredients
3. Bring up to 90-95% of final volume with MilliQ water
4. pH to 7.4 (use HCl)
5. Bring up to final volume; store at 4°C

DNA Extraction

1. Turn water bath on and heat up to 95°C
2. Determine volume of samples
3. Add appropriate volume of TNF buffer to bring total volume of samples to 0.6mL
4. Add 0.2mL 40% TCA to all tubes, for a final concentration of 10% TCA
5. Place tubes on ice for 10 minutes
6. Spin tubes at 1428xg, 10 minutes
7. Discard supernatant with Pasteur pipette
8. Add 0.6mL 10% TCA to all tubes
9. Place tubes in 95°C water bath for 30 minutes
10. Place tubes on bench top, then cool on ice for at least 5 minutes
11. Spin all tubes at 1428xg, 10 minutes
12. Save supernatant by transferring to 1.5mL eppendorf tube
13. Store sample at 4°C or assay for DNA right away

DNA Assay

1. Turn water bath on and heat up to 95°C
2. Make the following standards by diluting the stock standard (500µg/mL) with TNE buffer
    a. 500µg/mL (stock)
    b. 250µg/mL
    c. 125µg/mL
    d. 62.5µg/mL
    e. 31.25µg/mL
    f. 15.625µg/mL
    g. 7.8125µg/mL
3. Transfer 1mL of standard to appropriate cuvettes (do in duplicate)
4. Bring volume of sample to be analyzed up to 1mL with TNE buffer and transfer to the appropriate cuvette
5. When the water bath is up to temperature, add 2mL diphenylamine reagent to all cuvettes (be sure to use a glass pipette)

6. Mix well by covering tubes with parafilm and inverting twice
7. Place tubes in rack in water bath; place glass plate over tubes to prevent evaporation
8. Let sit in water bath for 10 minutes, or until visible color change in standards is noted
9. Place tubes on bench top for 5 minutes or until completely cool
10. Transfer to cuvettes and read at 600nm