

The Sertoli-spermatid junctional complex adhesion strength is affected *in vitro* by Adjudin

Katja M. Wolski¹, Dolores D. Mruk² and Don F. Cameron¹

¹Department of Anatomy, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC6, Tampa, FL 33612; ²Center for Biomedical Research, Population Council, 1230 York Ave., New York, NY 10021

Running head: Adjudin and the Sertoli-spermatid junction

Contact:

Katja M. Wolski

Department of Anatomy

University of South Florida College of Medicine

12901 Bruce B. Downs Blvd., MDC6

Tampa, FL 33612-4799

Phone: 813.974.9434

Fax: 813.974.2058

E-mail: kwolski@hsc.usf.edu

Abstract

The actin based cell-cell adherens junction (AJ) between the Sertoli cell and the germ cell in the mammalian testis is important not only in mechanical adhesion of the cells, but in the morphogenesis and differentiation of the germ cells. The Sertoli ectoplasmic specialization (ES), a specialized type of AJ, is associated with Sertoli-spermatid binding and is important in cell-cell adhesion in the seminiferous epithelium. Abnormal or absent Sertoli ESs have been associated with step-8 spermatid sloughing and oligospermia in conditions associated with reduced fertility potential. The reproductive hormones, follicle stimulating hormone (FSH) and testosterone (T), also have been shown to play a role in the regulation of binding of spermatids at the Sertoli-spermatid junctional complex (STJC). Adjudin [1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide] is a potential male contraceptive and is thought to exhibit its contraceptive effects by interrupting the STJC. It has been shown that this compound induces reversible germ cell loss from the seminiferous epithelium, particularly elongating/elongate/round spermatids and spermatocytes. Using a micropipette pressure transducing system (MPTS) to measure the force needed to detach step-8 spermatids from Sertoli cells, this study examined the strength of the STJC in Sertoli-spermatid cocultures in the presence of Adjudin (1 ng/ml, 50 ng/ml, 125 ng/ml, or 500 ng/ml in EtOH) and hormones [FSH (0.1 µg/ml, NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1), T (100 nM)] to optimize *in vitro* binding. The average forces required to detach the spermatids from the underlying Sertoli cells in the presence of 1 ng/ml, 50 ng/ml, 125 ng/ml and 500 ng/ml Adjudin were 18.2×10^{-10} pN, 14.3×10^{-10} pN, 7.74×10^{-10} pN and 6.51×10^{-10} pN, respectively. The average force required to detach step-8 spermatids in the presence of vehicle only (control) was 19.0×10^{-10} pN. A significant difference for Adjudin concentrations at or above 125 ng/ml was determined by one-way ANOVA ($p < 0.05$). These data confirm that Adjudin is effective in reducing the strength of the STJC, identifying Adjudin as a potential contraceptive agent in the male by inducing spermatid sloughing and therefore oligospermia.

Key words: ectoplasmic specialization, testis, micropipette, adherens junction, male contraception

Introduction

Spermatogenesis, the process in which germ cells undergo mitosis and meiosis to become elongated spermatids (sperm) (Leblond et al., 1963), occurs throughout the reproductive life of the male. Present in the seminiferous epithelium at any given point in time are several generations of germ cells in different stages of maturation (Courot et al., 1970). Various types of junctions between these developing germ cells and Sertoli cells exist throughout spermatogenesis, including the highly specialized ectoplasmic specialization (ES) found between Sertoli cells and germ cells (Russell, 1977a).

The ES is an apical cytoskeletal structure of the Sertoli cell associated with Sertoli-spermatid binding at the adherens junction (AJ) (Russell, 1977b; Russell, 1980). This structure is important in cell-cell adhesion in the seminiferous epithelium, ensuring the retention of spermatids as they mature into spermatozoa. It is believed that the ES forms to strongly anchor the step-8 spermatid to the seminiferous epithelium. In the rat, ESs are first seen at Stage VIII of spermatogenesis, the time when the step-8 spermatid appears, and are present at the AJ until appropriate release of the step-19 spermatid. Inappropriate release of spermatids (i.e., spermatid sloughing) is related to abnormal ES structure and oligospermia (O'Donnell et al., 1996; O'Donnell et al., 2000). A reduction of mature sperm in semen (Russell et al., 1988; Boekelheide et al., 1989; O'Donnell et al, 1996; O'Donnell et al, 2000) and conditions associated with oligospermia are associated with structurally abnormal or absent Sertoli ESs (Cameron and Griffin, 1998).

A number of health-related conditions are associated with reduced fertility potential and oligospermia in men, including varicocele (Cameron and Snyder, 1980), hyperprolactinemia (Cameron et al., 1984), diabetes (Murray et al., 1983), and idiopathic oligospermia (Cameron and Griffin, 1998). These conditions all are associated with reduced sperm in the semen, i.e., oligospermia, and ultrastructural pathology unique to the junctional apparatus of the seminiferous epithelium (Cameron and Griffin, 1998). Cap stage spermatids in the human (step-8 spermatids in the rat) are presumed to be tightly anchored to the seminiferous epithelium at a Sertoli cell AJ, which includes the unique Sertoli ectoplasmic specialization (Russell, 1977b; Russell, 1980). In both *in vitro* and *in vivo* observations of experimental animal models, disruption of this junction results in spermatid sloughing and subsequent oligospermia (Russell et al, 1988; Boekelheide et al, 1989; O'Donnell et al, 1996; O'Donnell et al, 2000).

Adjudin, formerly known as AF-2364 (1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide), depletes seminiferous tubules of germ cells (Cheng et al., 2001; Grima et al., 2001). By day 14 of administration of Adjudin, adult seminiferous tubules are found nearly devoid of elongated and round spermatids and spermatocyte numbers have been reduced, with no significant effect on reproductive hormone levels (Cheng et al., 2001). Although speculated, it is not yet been determined whether adjudin works at the level of the adherens junction or the ES.

This project was designed to measure the strength of junctions between step-8 spermatids and Sertoli cells in the presence of various concentrations of Adjudin. To do this, a micropipette pressure transducing system was used to measure the force needed to detach step-8 spermatids from Sertoli cells (Wolski et al., 2005) in the presence of Adjudin (0 ng/ml, 1 ng/ml, 50 ng/ml, 125 ng/ml, or 500 ng/ml in EtOH) (Siu et al., 2003) and reproductive hormones (follicle stimulating hormone and testosterone). It is hypothesized that Adjudin at higher concentrations will disrupt the STJC and cause reduced binding strength between the Sertoli cell and step-8 spermatid.

Methods

Sertoli and germ cells were isolated from Sprague-Dawley rats, as previously described (Cameron et al., 1987). Sixteen- to seventeen-day-old rats were used for Sertoli cell isolation, and adult rats were used for germ cell isolation.

Sertoli cell isolation, culture, and pre-treatment: Briefly, testes were excised from prepubertal male rats, and the parenchyma was digested using routine sequential enzymatic treatments with trypsin (0.25%, Sigma) and collagenase (0.20%, BD). Isolated cells were plated to near confluence on 13 mm round glass coverslips coated with 1:3 Matrigel (BD) in culture medium in 24-well cell culture dishes. Cultures were incubated in DMEM:F12 medium [supplemented with 0.01 mol/L retinol (Sigma), 1000 μ l/100 ml ITS (BD), 500 μ l/500 ml gentamicin (Sigma) and 5 ml/500 ml antibiotic/antimycotic (Cellgro)] at 39°C in a humidified incubator with 5% CO₂-95% air for 48 hrs to expedite the removal of contaminating germ cells. After the 48 hr pre-incubation, the cultures were exposed to a 20 mM Tris-HCl buffer for 2.5 min to hypotonically lyse any remaining germ cells, then incubated in supplemented DMEM:F12 at 33°C in a humidified incubator with 5% CO₂-95% air for 24 hrs. After the 24 hr incubation, the media was

replaced with supplemented DMEM:F12 containing 0.06 µg/ml follicle stimulating hormone (FSH; NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1) and 100 nM testosterone (T; Sigma) to optimize *in vitro* Sertoli-spermatid binding. These pre-treated Sertoli cell cultures were used in the coculture experiments.

Round spermatid isolation and unit gravity velocity sedimentation: Pre-step 9 spermatids (round spermatids) were isolated from an adult male rat testis. Briefly, the decapsulated adult testis was digested with 0.10% collagenase (Gibco; 37°C, 80 oscillations/min, 30 min) to separate seminiferous tubules from the testicular interstitial tissue. The washed seminiferous tubules then were digested with 0.25% trypsin (Sigma; 37°C, 90 oscillations/min, 15 min) to separate the peritubular cells from the seminiferous epithelium and to expedite the release of germ cells from the seminiferous epithelium. A 0.20% trypsin inhibitor solution (Sigma) was added to terminate the trypsin reaction. The resulting cell suspension (mixed germ cells and Sertoli cells) was resuspended in 25 ml McCoy's media + 0.5% BSA.

Using sterile technique, the gradient chambers on a STA-PUT velocity sedimentation cell separator were filled with the appropriate McCoy's + BSA medium (2% and 4% BSA), and a linear gradient (2-4%) was built under the cell suspension, at the loading rate initially at 10 ml/min. After 20 minutes, the rate was increased to 40 ml/min. Eighty minutes prior to the end of the collection time (4 h), media with germ cell fractions were collected using a Fractomat automatic fraction collector (10 ml/vial at 160 drops/min). Round spermatids (pre-step 9) were identified by phase contrast microscopy and pooled, washed, and resuspended in McCoy's media. The number of cells in the spermatocyte and spermatid fractions were counted by hemocytometric analysis and assayed for viability by trypan blue exclusion.

Sertoli-germ cell coculture: Approximately 400,000 isolated germ cells (round spermatid-enriched) were added directly to the pre-treated Sertoli cell enriched monocultures. The Sertoli-germ cell cocultures were incubated in a humidified chamber at 33°C with 5% CO₂-95% air for 36 hrs with 0.06 µg/ml FSH + 100 nM T to optimize Sertoli-spermatid binding as previously described (Cameron and Muffly, 1991; Cameron et al., 1993).

Addition of Adjudin to the coculture: After 30 hrs of incubation, Vehicle 1 (2.5 µl EtOH) was added to one column of the 24-well plate and incubated for 1 hr at 33°C. After the 1 hour incubation time, the next column received 1 ng/ml Adjudin in 2.5 µl EtOH and incubated for 1 hr at 33°C. This continued

with the remaining concentrations of Adjudin (50 ng/ml, 125 ng/ml and 500 ng/ml) (Siu et al, 2003) and ended with Vehicle 2 (same as Vehicle 1) to ensure that time was not the factor affecting the junction.

Measurement of junctional strength using a micropipette pressure transducing system (MPTS):

The Sertoli-germ cell cocultures were imaged on an inverted interference contrast microscope (Axiovert 100, Zeiss) with a 20x objective. The microscope was fitted with the MPTS, as previously described (Wolski et al, 2005). After the 1 hr incubation with the treatment, the cover slips were washed 5x by gentle pipetting with supplemented DMEM:F12 + FSH and T (without Adjudin or EtOH). Cover slips containing the Sertoli-germ cell cocultures were carefully removed from the well. A step-8 spermatid were identified as a 10 μm round cell containing an eccentric nucleus, as previously described (Leblond and Clermont, 1952; Cameron and Muffly, 1991). The detachment of individual step-8 spermatids from Sertoli cells and subsequent force measurement and analysis was performed as previously described (Wolski et al, 2005). Briefly, pressure at the tip of a 10 μm -diameter micropipette was controlled by a system consisting of two water reservoirs and a pressure transducer connected between the two reservoirs. To detach spermatids from Sertoli cells, the glass micropipette tip was brought into close proximity to the unbound cell surface of the spermatid, and the hydrostatic pressure required to detach it from the underlying Sertoli cell monolayer was recorded on the transducer. The recorded pressure (in $\text{cm-H}_2\text{O}$) was used to calculate force via the equation $F = \Delta P \cdot \pi R^2 p$, where F (pN) is the force on a static cell, ΔP is the suction pressure ($\text{N}/\mu\text{m}^2$), and $\pi R^2 p$ is the cross sectional area of the pipette (μm^2). To convert the pressure reading received in $\text{cm-H}_2\text{O}$ to $\text{N}/\mu\text{m}^2$, for use in the above equation, the conversion factors $1 \text{ cm-H}_2\text{O} = 98.06 \text{ Pa}$ and $1 \text{ Pa} = \text{N}/\text{m}^2$ were used, since the international unit of force is Newtons ($1 \text{ N} = 1 \text{ kg}\cdot\text{m}/\text{s}^2$), and the international unit of pressure is Pascal (Pa). Each detachment event (a maximum of 4) consisted of a 5 second suction pressure interval. If the germ cell did not dissociate, the detachment effort was abandoned, and the last pressure reading was recorded.

Viability/cytotoxicity assays: Sertoli cell cultures were prepared from 20-day-old rat testes by sequential enzymatic treatments as described (Cameron et al, 1987), and cells were plated at high-density ($0.5 \times 10^6 \text{ cells}/\text{cm}^2$) on Matrigel™ (diluted 1:7 with Ham's F-12 Nutrient Mixture and Dulbecco's Modified Eagle's Medium [F-12/DMEM], 1:1; Sigma)-coated Nunclon 24-well dishes in F-12/DMEM supplemented with 10 $\mu\text{g}/\text{ml}$ bovine insulin, 5 $\mu\text{g}/\text{ml}$ human transferrin, 10 $\mu\text{g}/\text{ml}$ bacitracin, 2.5 ng/ml

EGF, 0.06 $\mu\text{g/ml}$ FSH and 100 nM T. To obtain Sertoli cells with a purity greater than 98%, cultures were hypotonically treated. Media were replaced every 24 hr thereafter, and Sertoli cells were incubated for an additional 3 days. This was followed by the isolation of germ cells from 90-day-old rat testes as previously described (Aravindan et al., 1996; Aravindan et al., 1997). In this experiment, germ cell preparations were exposed to successive glass wool filtration steps, and thus, consisted of spermatogonia, spermatocytes, round and elongating spermatids when examined microscopically. Germ cells were added directly to Sertoli cell cultures at a Sertoli:germ cell ratio of 1:3 and cocultured for 36 hr (Mruk et al., 1997). Thereafter, cocultures were rinsed twice with media to remove unbound germ cells and increasing concentrations of Adjudin (1, 50, 125 and 500 ng/ml, and 1 $\mu\text{g/ml}$) were added. Sertoli-germ cell cocultures were incubated for 0, 1, 3, 6 and 12 hr. These cocultures were then used for viability/cytotoxicity assays. Because viable cells are characterized by the presence of intracellular esterase activity, this assay measured the ability of cells to enzymatically convert non-fluorescent, cell-permeable calcein AM to fluorescent calcein. Briefly, media was removed from Sertoli-germ cell cocultures and cells gently rinsed with media. Calcein AM (~2-5 μM , prepared in media or PBS, pH 7.4 prior to immediate use to prevent hydrolysis; Invitrogen) was added to Sertoli-germ cell cocultures and incubated briefly at 37 °C or room temperature. Fluorescence was quantified at 10-15 min intervals for up to 60 min at 485 nm_{EX} and 535 nm_{EM} using a Tecan GENios fluorescence plate reader. Controls consisted of Sertoli-germ cell cocultures cultured in the absence of Adjudin and in the presence of vehicle (ethanol:DMSO, 1:1 dilution). Non-viable Sertoli-germ cell cocultures, which lacked the ability to enzymatically convert calcein AM to calcein, were prepared by treating cells with 75% ethanol (30 min) or 0.5% saponin (10 min).

Results

The mean force required to detach step-8 spermatids from Sertoli cells in the optimized Sertoli-germ cell *in vitro* binding model in the presence of various concentrations of Adjudin was determined following multiple measurements acquired from the modified MPTS. The mean force necessary to detach step-8 spermatids from Sertoli cells (i.e., Sertoli-spermatid junctions with ES) in the presence of Vehicle 1 (at the start of the measurement process) and Vehicle 2 (at the end of the measurement process) was

18.03x10⁻¹⁰ pN (SE=1.263x10⁻⁶, n=16) and 19.92x10⁻¹⁰ pN (SE=1.149x10⁻⁶, n=16). The average for the two Vehicle groups was 19.0x10⁻¹⁰ pN. In the presence of 1 ng/ml, 50 ng/ml, 125 ng/ml and 500 ng/ml Adjudin, the mean forces required to detach step-8 spermatids from the underlying Sertoli cell monoculture were 18.2x10⁻¹⁰ pN (SE=1.383x10⁻⁶, n=16), 14.3x10⁻¹⁰ pN (SE=1.412x10⁻⁶, n=16), 7.74x10⁻¹⁰ pN (SE=1.122x10⁻⁶, n=16) and 6.51x10⁻¹⁰ pN (SE=1.750x10⁻⁶, n=10), respectively (Figure 1). A one-way ANOVA determined a significant difference for Adjudin concentrations at or above 125 ng/ml, where p<0.05.

Viability/cytotoxicity assays demonstrated that the viability of Sertoli-germ cell cocultures was not affected when these cells were incubated with increasing concentrations of Adjudin for up to 12 hr (Figure 2). Time points beyond 12 hr were not examined because higher doses of Adjudin (500 ng/ml and 1 µg/ml) perturb Sertoli-germ cell adhesion, resulting in a decrease in cell number in these wells.

Discussion

It is hypothesized that Adjudin works at the level of the AJ and possibly the ES in the seminiferous tubules, since depletion of round and elongated spermatids is seen in rats after administration of this potential male contraceptive (Cheng et al, 2001; Grima et al, 2001; Cheng et al., 2005). However, cell adhesion was not compromised in other organs such as the brain, liver and kidney when this drug was administered by gavage, inter-peritoneal or intramuscular injection (Cheng et al, 2005) nor was the hypothalamus-pituitary-testicular axis affected at doses that were effective to induce male infertility (Grima et al, 2001). Additionally, administration of Adjudin (50 mg/kg b.w., 3 doses administered every 2 days) to pups (e.g., 5-25 days of age) did not effect the integrity of Sertoli-germ cell adherens junctions (Mruk and Cheng, 2004), suggesting that the apical ES is an initial target. Upon metabolic removal of Adjudin from virtually all organs by 48 hr, spermatogenesis began to resume progressively, and by day ~100 the testes of treated animals were indistinguishable from controls (Grima et al, 2001). However, it was not known whether this compound actually affects the strength of the STJC.

We have, for the first time, determined that the strength necessary to detach step-8 spermatids from a Sertoli cell monolayer is reduced by specific doses of Adjudin *in vitro*, indicating a functional alteration of the STJC. The data presented confirm the hypothesis that this compound, at higher

concentrations, disrupts the STJC, causing weaker binding between the Sertoli cell and step-8 spermatid. Only the STJC between step-8 spermatids and Sertoli cells, which contains the ES, was tested. It is not known whether Adjudin affects the strength of desmosome-like junctions that are present, for example, between Sertoli and pachytene spermatocytes. Recent studies have suggested that Adjudin affects the ES by activating RhoB within hours of administration. This in turn activated ROCK, LIMK1 and cofilin, which perturbed actin cytoskeleton dynamics and resulted in germ cell detachment (Lui et al., 2003). Moreover, β 1 integrin, which is predominantly located at the apical ES, was also shown to be up-regulated following Adjudin treatment, further activating the FAK/PI 3-kinase/p130Cas/MAP kinase (Siu et al, 2003) pathway. Though the exact protein complex acting as the receptor for Adjudin has not yet been defined, it is thought that through these signaling pathways, changes in the polymerization and depolymerization of actin at the ES lead to a depletion of germ cells from the seminiferous epithelium, in particular round and elongating spermatids (Cheng et al, 2005). It should be noted that Adjudin is a chemical entity that shares structural similarities with Ionidamine [1-(2,4)-dichlorobenzyl-1H-indazole-carboxylic acid], which is known to severely damage stress fibers (e.g., actin filaments) in Sertoli cells (De Martino et al., 1981; Silvestrini et al., 1984). Likewise, preliminary studies have shown that Adjudin can induce extensive remodeling of the actin cytoskeleton in these cells (Mruk and Cheng, unpublished observations). What remains to be determined, however, is why Sertoli cell actin at the apical ES is sensitive to Adjudin's effects when this protein is a constituent of virtually all cell types. Certainly, other upstream regulators of RhoB activity, as well as additional signaling cascades, are likely to be involved, and their identification will help in determining why the apical ectoplasmic specialization is a primary target for Adjudin-mediated restructuring in the testis.

Results from this study show that the junctional strength between Sertoli cells and step-8 spermatids is reduced by Adjudin *in vitro*, supporting the potential use of this chemical as a male contraceptive.

References

- Aravindan GR, Mruk D, Lee WM, Cheng CY. Identification, isolation, and characterization of a 41-kilodalton protein from rat germ cell-conditioned medium exhibiting concentration-dependent dual biological activities. *Endocrinology*. 1997;138:3259-3268.
- Aravindan GR, Pineau CP, Bardin CW, Cheng CY. Ability of trypsin in mimicking germ cell factors that affect Sertoli cell secretory function. *J Cell Physiol*. 1996;168:123-133.
- Boekelheide K, Neely MD, Sioussat TM. The Sertoli cell cytoskeleton: a target for toxicant-induced germ cell loss. *Toxicol Appl Pharmacol*. 1989;101:373-389.
- Cameron DF, Griffin FC. Ultrastructure of Sertoli-germ cell interactions in the normal and pathologic testis. RJ M-GF, ed. In: *Male Reproduction: A multidisciplinary overview*. Spain: Churchill Communications Europe España, 1998:229-242.
- Cameron DF, Muffly KE. Hormonal regulation of spermatid binding. *J Cell Sci*. 1991;100 (Pt 3):623-633.
- Cameron DF, Muffly KE, Nazian SJ. Reduced testosterone during puberty results in a midspemiogenic lesion. *Proc Soc Exp Biol Med*. 1993;202:457-464.
- Cameron DF, Murray FT, Drylie DD. Ultrastructural lesions in testes from hyperprolactinemic men. *J Androl*. 1984;5:283-293.
- Cameron DF, Snyder FE. The blood-testis barrier in men with varicocele: a lanthanum tracer study. *Fertil Steril*. 1980;34:255-258.
- Cameron DF, Wyss HU, Romrell LJ. Alterations of androgen binding protein (ABP) in Sertoli/spermatid co-cultures with varying glucose concentrations. Orgebin-Crist MC, Danzo BJ, eds. In: *Cell Biology of the Testis and Epididymis*. New York: NY Acad Sci, 1987:448-451.
- Cheng CY, Mruk D, Silvestrini B, Bonanomi M, Wong CH, Siu MK, Lee NP, Lui WY, Mo MY. AF-2364 [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide] is a potential male contraceptive: a review of recent data. *Contraception*. 2005;72:251-261.
- Cheng CY, Silvestrini B, Grima J, Mo MY, Zhu LJ, Johansson E, Saso L, Leone MG, Palmery M, Mruk D. Two new male contraceptives exert their effects by depleting germ cells prematurely from the testis. *Biol Reprod*. 2001;65:449-461.

- Courot M, Hochereau-de-Reviere M, Ortavant R. Spermatogenesis. A.D. J, W.R. G, N.L. V, eds. In: The Testis. New York: Academic Press, vol. 1, 1970:339-432.
- De Martino C, Malcorni W, Bellocchi M, Floridi A, Marcante ML. Effects of AF 1312 TS and lonidamine on mammalian testis. A morphological study. *Chemotherapy*. 1981;27 Suppl 2:27-42.
- Grima J, Silvestrini B, Cheng CY. Reversible inhibition of spermatogenesis in rats using a new male contraceptive, 1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide. *Biol Reprod*. 2001;64:1500-1508.
- Leblond CP, Clermont Y. Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfuric acid technique. *Am J Anat*. 1952;90:167-215.
- Leblond CP, Steinberger E, Roosen-Runge EC. Spermatogenesis. CG H, ed. In: *Mechanisms Concerned With Conception*. New York: MacMillan Co, 1963:1-72.
- Lui WY, Lee WM, Cheng CY. Sertoli-germ cell adherens junction dynamics in the testis are regulated by RhoB GTPase via the ROCK/LIMK signaling pathway. *Biol Reprod*. 2003;68:2189-2206.
- Mruk D, Zhu LJ, Silvestrini B, Lee WM, Cheng CY. Interactions of proteases and protease inhibitors in Sertoli-germ cell cocultures preceding the formation of specialized Sertoli-germ cell junctions in vitro. *J Androl*. 1997;18:612-622.
- Mruk DD, Cheng CY. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr Rev*. 2004.
- Murray FT, Cameron DF, Orth JM. Gonadal dysfunction in the spontaneously diabetic BB rat. *Metabolism*. 1983;32:141-147.
- O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM. Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. *Biol Reprod*. 1996;55:895-901.
- O'Donnell L, Stanton PG, Bartles JR, Robertson DM. Sertoli cell ectoplasmic specializations in the seminiferous epithelium of the testosterone-suppressed adult rat. *Biol Reprod*. 2000;63:99-108.
- Russell L. Observations on rat Sertoli ectoplasmic ('junctional') specializations in their association with germ cells of the rat testis. *Tissue Cell*. 1977a;9:475-498.
- Russell L. Sertoli-germ cell interrelations: a review. *Gamete Res*. 1980;3:179-202.

- Russell LD. Desmosome-like junctions between Sertoli and germ cells in the rat testis. *Am J Anat.* 1977b;148:301-312.
- Russell LD, Goh JC, Rashed RM, Vogl AW, Weber JE, Wong V, Peterson RN, Lee IP, Ettlin R, Malone JP, Russell L. The consequences of actin disruption at Sertoli ectoplasmic specialization sites facing spermatids after in vivo exposure of rat testis to cytochalasin D. *Biol Reprod.* 1988;39:105-118.
- Silvestrini B, Palazzo G, De Gregorio M. Lonidamine and related compounds. *Prog Med Chem.* 1984;21:110-135.
- Siu MK, Mruk DD, Lee WM, Cheng CY. Adhering junction dynamics in the testis are regulated by an interplay of beta 1-integrin and focal adhesion complex-associated proteins. *Endocrinology.* 2003;144:2141-2163.
- Wolski KM, Perrault C, Tran-Son-Tay R, Cameron DF. Strength measurement of the Sertoli-spermatid junctional complex. *J Androl.* 2005;26:354-359.

Figure 1.

Adjudin Concentration vs Sertoli-Spermatid Strength

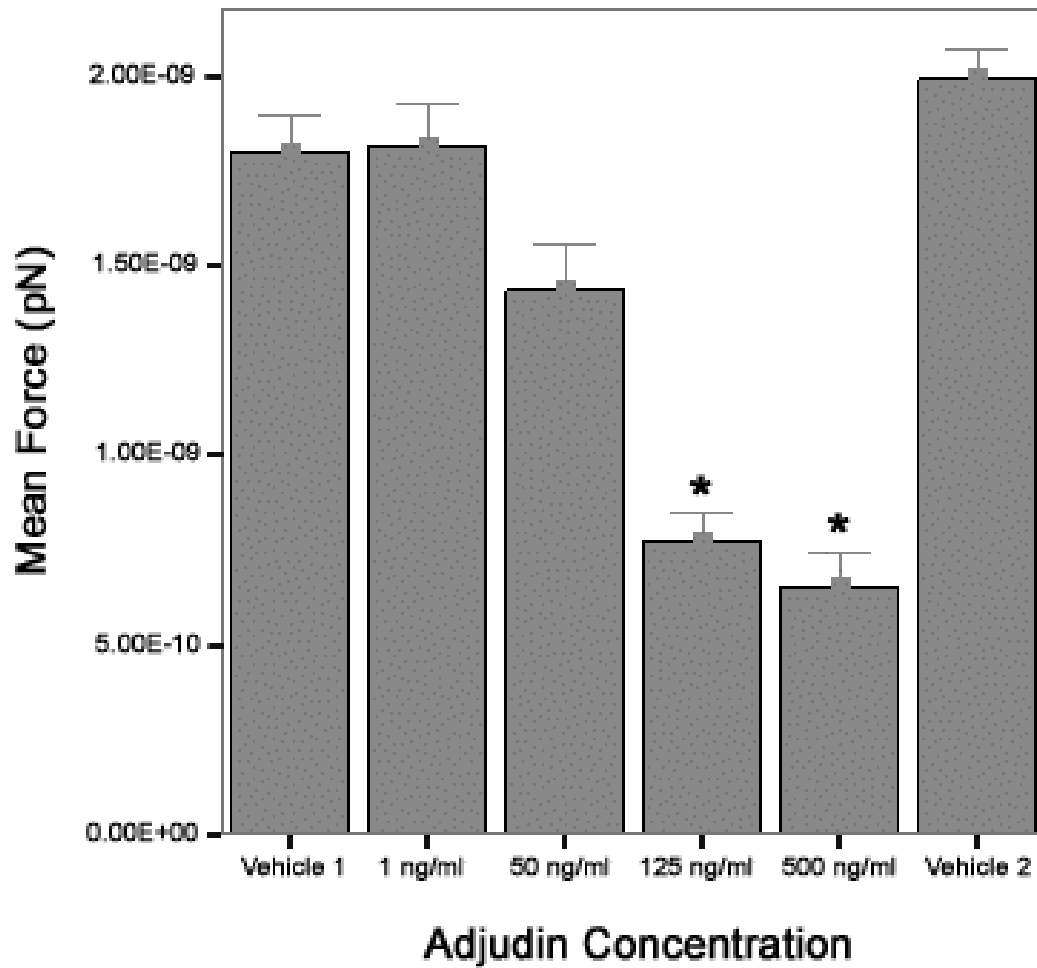


Figure 1. Bar graph displaying the effect of Adjudin on the mean force (in piconewtons, pN) required to detach step-8 spermatids from Sertoli cells in the optimized Sertoli-spermatid coculture binding model. * indicates a significant difference, as determined by one-way ANOVA.

Figure 2.

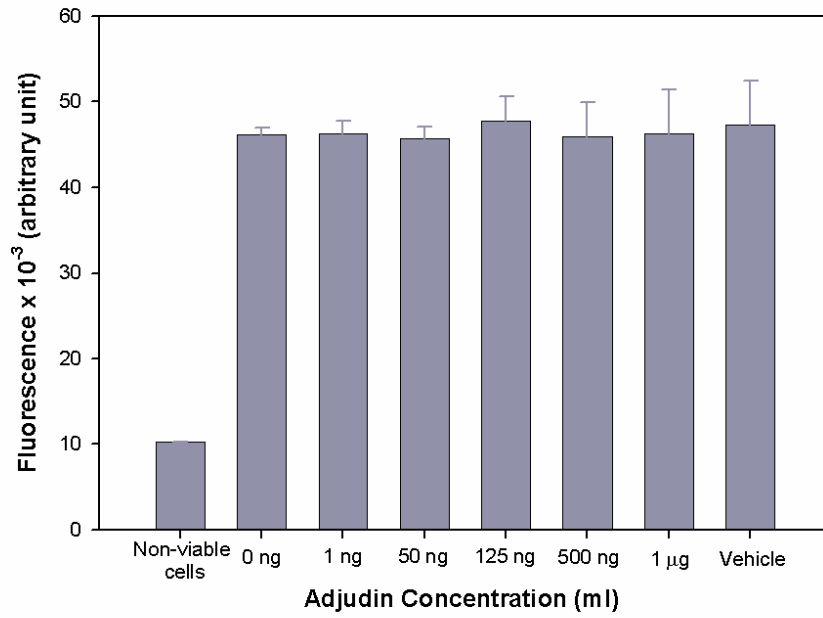


Figure 2. Bar graph illustrating that increasing concentrations of Adjudin (0, 1, 50, 125 and 500 ng/ml, and 1 µg/ml) at 12 hr post-treatment had no effect on the viability of Sertoli-germ cell cocultures when compared to controls (0 ng/ml Adjudin and vehicle).

Adjuvins Concentration vs Bertoli-Spermatid Strength

