Sumoylation Inhibition in Sertoli Cells Reduces Cell Viability via Apoptosis

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Abstract

Male Infertility affects 15% of couples worldwide and is a contributing factor to about 50% of cases of infertility. While sometimes attributed to certain environmental factors and genetic mutations, many cases of infertility require a deeper understanding of spermatogenesis and the necessary enzymes that promote its progression in order to diagnose and treat effectively. Recent studies have explored the unique SUMO (small ubiquitin-like modifier) protein and how it modifies post-translational proteins by sumoylation in a variety of ways. Our lab has focused on its role within the various cells in the male testes. In this study, we sought to determine importance of sumoylation specifically in the supportive somatic cells found in the testes, known as sertoli cells. Sertoli cells are known to provide nutritional, mechanical and immune support toward differentiating cells during spermatogenesis. In each experiment, we treated sertoli cell lines with the sumoylation inhibitor, ginkgolic acid at varying concentrations and analyzed the resultant cell fate. A WST-1 viability assay revealed that sertoli cells shut down when sumoylation was inhibited. Furthermore, we identified apoptosis as the particular mode of cell death initiated by these cells lacking sumoylation ability. Sumoylation, however, was not proven necessary for the proliferation of sertoli cells, which was not affected by the addition of ginkgolic acid. Future studies will explore more specific identification of SUMO targets in sertoli cells and which directly activate apoptosis.

I. Introduction

It has been estimated that 15% of couples around the world struggle with infertility. While 20-30% of those cases have been attributed solely to male infertility, a total of 50% of those challenges can at least be partially contributed by the male reproductive system (Agarwal *et al*, 2015). Causes of male infertility range from environmental factors and endocrine problems to obstructive syndromes and poor sperm motility, yet it has been estimated that many cases are treatable. The precise biological mechanisms which cause male infertility must be analyzed and identified in order to design fertility treatments. Numerous stages within and pathways involved in the development of sperm during spermatogenesis have been recognized as possible contributing factors to infertility (Cheng and Mruck, 2010) and require further research in order to understand what processes may be impaired and which particular proteins are involved in those changes. This will allow us to develop treatments to inhibit these aberrant cellular processes and promote fertility.

II. Background

A. Spermatogenesis

The process of development and differentiation of male sperm cells is known as spermatogenesis. Sperm cells originate and proliferate in the male reproductive organ, the testes, within its coiled tubes called seminiferous tubules. Beginning their development as spermatogonia, these germ cells proliferate via mitosis, multiply by meiosis into primary and

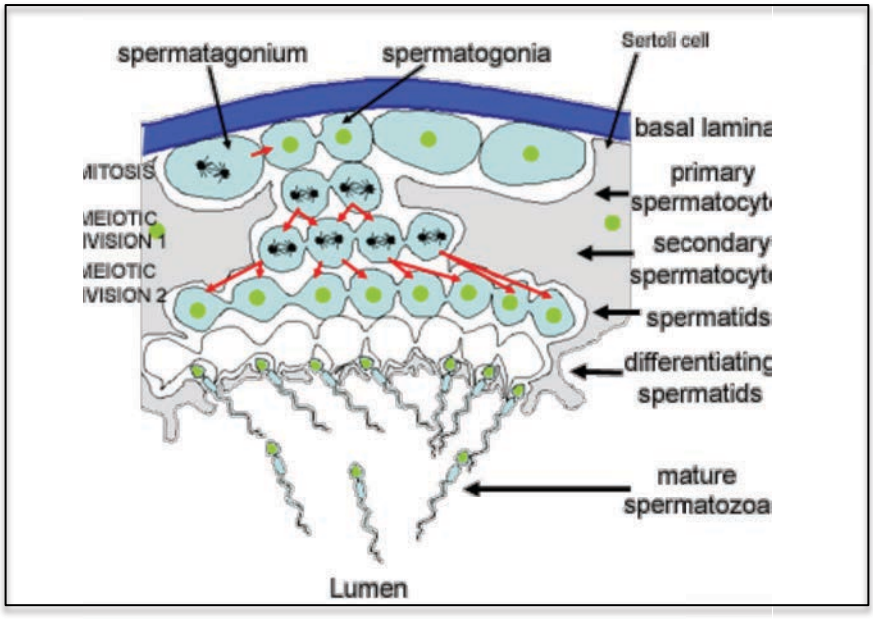
secondary spermatocytes before finally differentiating via spermiogenesis into spermatids. The spermatids become motile and fully matured in the epididymis and are stored there until ejaculation and potential fertilization of the female ovum.

Sperm cells have the potential to be altered at each step during their development process. A method called *in vitro* spermatogenesis has been developed in order to artificially drive spermatogenesis in patients who suffer from oligospermia, defined as severe deficiency in sperm production or azoospermia, defined as those who produce no usable sperm at all (Ibtisham *et al* 2017). This method has allowed us to evaluate the many stages of spermatogenesis and condense the process into its more necessary steps, which can help us clarify the most essential parts. By employing this technique, testicular biopsies can allow clinicians to extract stem cells which can then be differentiated into the necessary cells at the stage of spermatogenesis that has been impaired in that individual to be re-implanted into the individual or developed fully *in vitro* and can be used to fertilize an egg *in vitro* as well (Ibtisham *et al* 2017). This method represents one practical application of a deep assessment and analysis of the intricacies of spermatogenesis. We may also use this research in order to design drugs that can target problems in the progression of spermatogenesis within a patient's own reproductive system to promote a patient's fertility and make natural conception a viable possibility.

B. Sertoli Cells

We have mentioned that the germ cells which continuously develop into sperm cells during many complex steps. However, there are a number of somatic cell types such as

leydig and sertoli which are needed for the successful progression of spermatogenesis. Sertoli cells in particular serve as essential support cells or nurse cells for the spermatogonia within the testes' seminiferous tubules, pictured in **figure 1**. Beyond the germ cells alone which differentiate into functional sperm cells, somatic sertoli cells serve as important mediators of spermatogenesis. Sertoli cells secrete proteins and other molecules necessary for sperm cell development and maturation, secure the blood-testis barrier (BTB), and facilitate adhesion between sertoli cells called tight junctions as well as between sertoli cells and germ cells via adhesion junctions. The secretion of molecules like lactate, cytokines and hormones provide nutritional and mechanical support to sperm cells, while the junctions between cells and those that form the BTB serve a protective role in the immune system (Ni *et al*, 2019).



matic diagram of seminiferous tubule during stages of
atogenesis. Sertoli cells are pictured on the sides of the
section, playing a supportive role for the dividing germ cells
n *et al* 1970).

A closer look at the various roles of sertoli cells highlights the key signaling pathways within spermatogenesis that prompt the proliferation of sertoli cells. Serving as the fewer somatic cells within the large population of germ cells within the male reproductive system, sertoli cells are driven by a plethora of cell signaling pathways. Some of the most researched pathways include the AMP-activated protein kinase signaling pathway (AMPK), the transforming growth factor-β (TGF- β)/Smad signaling pathway, and the mitogen-activated protein kinases (MAPK). Other common pathways involved include the follicle stimulating hormone (FSH)/adenylate cyclase/cyclic adenosine monophosphate (cAMP)/protein kinase A signaling pathway, the Hippo signaling pathway, the integrin mediated signaling pathway, as well as many others. The aforementioned pathways however, are uniquely recognized as main contributors to the proliferation of sertoli cells that support spermatogenesis, and specifically, AMPK and MAPK have been linked to the lactate production in sertoli cells, while MAPK alone has been found to mediate sertoli cell self-renewal (Ni *et al*, 2019).

While the interaction between the different pathways is not yet entirely understood, the specific roles of sertoli cells which have been found to be foundational in the maintenance of the male testes have been outlined. Sertoli cells seem to alter their morphology significantly during their lifetimes while they act to alter spermatids during each stage of the spermatogenic cycle (Griswold 2018).

C. Sumoylation

Several processes have the capability to alter and regulate spermatogenesis after genetic transcription has occurred, which are known as post translational modifications.

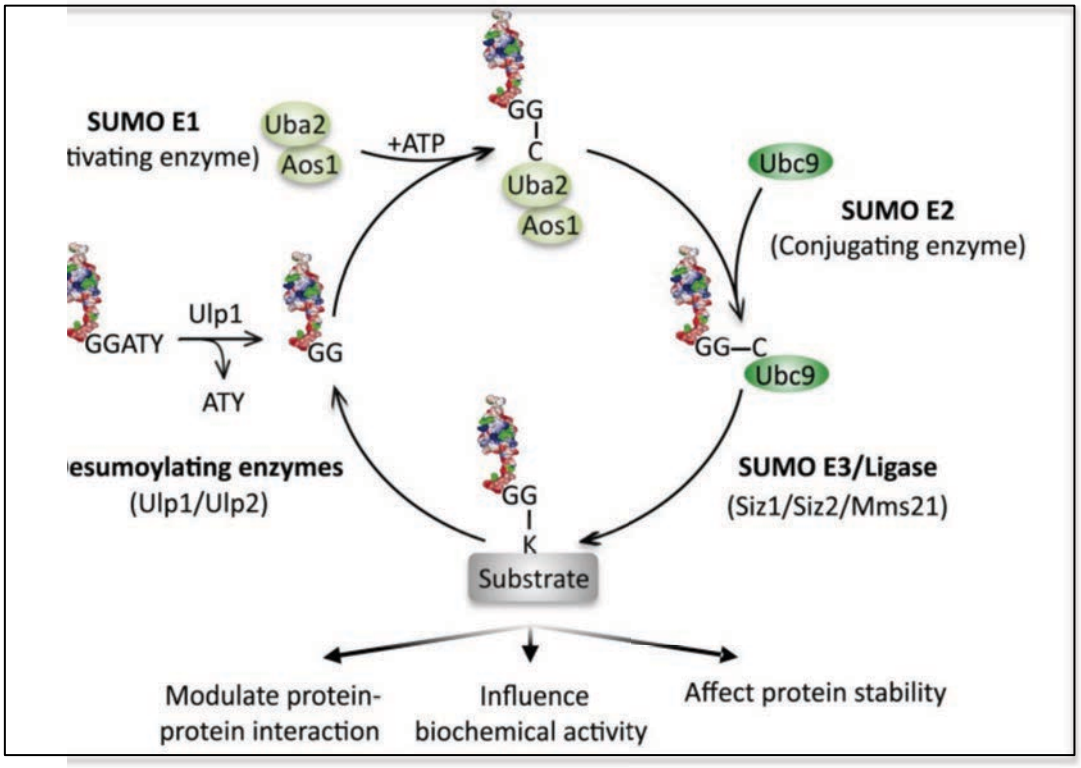
Aside from common modifier proteins such as methyltransferase, and ubiquitination, a more recently studied modifier protein is the SUMO protein, or small ubiquitin-like modifier. While SUMO proteins share a similar structure to ubiquitin, its amino acid sequence is different (Bayer *et al* 1998).

SUMO proteins have been studied across many somatic cell types and found to target many kinds of cellular proteins. Its functions have included DNA replication, transcription and translation, as well as cellular transport and RNA metabolism (Vigodner *et al* 2016). As its acronym describes, SUMO proteins' functions resemble those of ubiquitin in that they both signal proteins for necessary degradation. SUMO attachment can also affect a protein's localization, activation and other interactions (Kerscher 2007). Sumoylation can apparently also modify certain proteins without direct covalent attachment (Vigodner *et al* 2016).

Many enzymes have been identified during various parts of the modification process, known as the sumoylation cycle, demonstrated in **figure 2**. A SUMO protein is first cleaved from an inactive precursor by a protease which removes several c-terminal amino acids. SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligase protein (E3) then sequentially bind SUMO proteins to lysine residues upon cellular substrates, creating isopeptide bonds. Substrates can be altered rapidly by these enzymatic actions and changes in protein conformation and stability. SUMO bonding can also be reversed through desumoylation by cleaving the isopeptide bond by sentrin-specific proteases (SENPs) (Vigodner *et al* 2016, Cremona *et al* 2012).

The activating enzyme, E1 requires ATP to form a thioester bond with the cysteine residue of E2/Uba2. The SUMO conjugating enzyme, E2, or Ubc9 overlaps with the same binding spots of E1. A greater SUMO concentration was more associated with a SUMO-

Uba9 complex than an E1-Uba9 complex. The SUMO ligase protein, E3 was most recently discovered as essential to sumoylation mechanisms and plays a role in E2's conjugation process (Dohmen *et al* 2004).



Over the past approximately 20 years, scientists have been studying and decoding the intricacies of the SUMO protein and its unique functions. We have identified four different SUMO paralogs, numbered SUMO 1-4 . SUMOs 1,2, and 3 have been found on many tissues and seem to resemble each other, while SUMO 4 has been confined to the kidney, liver, and lymph nodes. Since SUMO-2 and SUMO-3 proteins have been found to share 97% of their identity, we use SUMO-2/3 to describe the isoform. SUMO-1 shares about 50% of the genes of SUMO-2/3. While both of these common SUMO proteins have been found to be involved in mitosis and the changes in chromosomal arrangement during its stages, SUMO-1 was

identified as binding to proteins like Ran-GAP1 and localizing them in the mitotic spindle, while SUMO-2/3 was mainly found in proteins migrating to centromeres and kinetochores (Cubaness-Potts *et al* 2015).

More recently, SUMO proteins have been identified specifically within cells in the male testes during spermatogenesis. An earlier study showed that SUMO-conjugating machinery was primarily involved in the necessary support of spermatogenesis and that SUMO-2/3 could act interchangeably with SUMO-1 to perform necessary functions (Zhang *et al* 2008). In our lab, numerous imaging and immunodetection methods have been used to isolate and identify SUMO targets in cells at all stages of spermatogenesis including spermatogonia, spermatocytes, spermatids and somatic cells within the testes (Vigodner *et al* 2016). In addition to localizing these SUMO protein modifications, we must devote future research toward identifying the full gamut of SUMO target proteins within the testes and the precise functions for which they alter them. This will help us determine how essential the process of sumoylation is and help us design how to simulate those important processes even when cellular SUMO proteins are inactivated or inhibited.

III. Goals

Our lab has previously studied and identified various target cells of sumoylation in the cycles of spermatogenesis. During my time in the Vigodner lab, I worked under the guidance of Dr. Stav Kemeny, seeking to understand the particular functions of sumoylation in the unique somatic sertoli cells which serve a crucial supportive role within the cells of the testes. In order to investigate the specific effects of sumoylation, we inhibited sumoylation in

cell lines in order to measure the viability of those sertoli cells, what mode of cell death they might encounter if unviable, and finally, whether inhibition of sumoylation could suppress the proliferation of sertoli cells. Each of these three goals would help address major questions in male infertility research, by determining the particular cellular processes that promote cell viability and growth when present and promote cell death when absent will help scientists design appropriate treatments to preserve male fertility and sperm count necessary to father children.

IV. Materials and Methods

A. Cell Lines

Mouse sertoli cell lines were used in all three of the experiments conducted. The cell lines, labeled 15P-1 (ATCC®, CRL-2618) were purchased from the American Type Culture Collection (ATCC) in Manassas, VA. The cell lines were grown in DMEM media, containing 5% fetal bovine serum (FBS, Life Technologies, 16140-071), 5% bovine growth serum (Fisher Scientific , SH30541.03), 1% penicillin/streptomycin (Life Technologies, 15140-122), and 0.5% Fungizone (Life Technologies , 15290-018) at 32° C with 5% CO_2.

The sertoli cell line was originally derived from primary mouse cells and cultured as cell lines in order to be maintained and studied over several days. The cells were maintained in their plates by being split, or trypsinized twice a week using trypsin/EDTA. Each plate well that was used in the experiments contained about 50,000 cells.

B. Inhibition of sumoylation by Ginkgolic Acid (GA)

In order to measure the viability of sertoli cells when their sumo proteins are not expressed and functional, we treated sertoli cells with a recognized sumoylation inhibitor in each of the experiments performed. Ginkgolic acid (GA) has been identified as an effective inhibitor of sumoylation both *in vitro* and *in vivo* by binding directly to enzyme 1 (E1) and preventing the formation of the E1-SUMO complex intermediate during the sumoylation cycle. GA was the first inhibitor discovered that specifically inhibited the E1 enzyme activity with SUMO proteins without affecting E1 in ubiquitin (Fukuda *et al*, 2009). This novel discovery allowed us to use GA as an isolated determinant of SUMO activity to the exclusion of other post-translational modifications.

In each of the experiments conducted, sertoli cell lines were treated with GA of concentrations varying from 50-200 μM for varying periods of time up to 24 hours, as indicated in each of the experimental **figures 3-5**. In each experiment, cells were contained in the same amount of DMSO, or dimethyl sulfoxide, a common cryopreservant as a control (Zu *et al*, 2019). Each of the experiments was performed in triplicates in order to maximize reliability of the resultant data.

C. WST-1 assay

The WST-1 assay was used to measure the viability of sertoli cells by spectrophotometry after cells were treated with GA. This useful test is reliable and sensitive in determining the viability and proliferative abilities of treated cells. WST-1 is a tetrazolium

salt which gets easily reduced to soluble purple dye, formazan when it reacts with glycolytic reducing agent NADH via an electron mediator through plasma membrane electron transport (Francoeur *et al*, 1996). The presence of pigmented formazan in solution indicates cell viability since the necessary reducing reagent to react and produce colored product is only present in metabolically active cells containing mitochondrial dehydrogenase enzymes. The precise machinery which cleave formazan from WST-1 involves the succinate-tetrazolium reductase system which belongs to viable cells containing mitochondria only. Therefore, concentration of formazan in solution directly corresponds to the number of viable cells in the well (Francoeur *et al*, 1996).

To measure the viability of sertoli cells, we seeded sertoli cell tissue culture we had been maintaining and splitting regularly in the wells of a 96-well plate, adding 100 µL of culture medium and final cell density of about 50,000 total cells. The plate was incubated at 37° C with 5% CO_2. The cells were then treated with varying concentrations of GA. The first column of six samples received no treatment as a control and DMSO alone was added to the second column of wells and DMSO added to the rest of the columns wells along with GA. Cells in columns 3,4,5 and 6 were treated with 50, 100, 125 and 150 µM of GA respectively. The cell plate was incubated for 24 hours. Subsequently, 10 µL of WST-1 reagent, from the WST-1 Cell Proliferation Assay Kit from Cayman Chemicals (item no. 10008883) was added to each of the wells and the plate was incubated for 4 hours at 37° C in a CO_2 incubator. The plate was then shaken on a shaker for 1 minute to allow thorough color distribution throughout the wells. Finally, the plate was placed in a multi-plate reader spectrophotometer and measured for absorbance in each well at a wavelength of 450 nm.

D. Western Blot

Two western blots were performed on the sertoli cell lines used in this study. The first western blot was used to detect apoptotic events in GA treated sertoli cells while the second was used to measure whether GA's inhibition of sumoylation in sertoli cells would affect cellular proliferation.

Western blotting materials were purchased from Sigma Aldrich (St. Louis, Missouri) unless otherwise specified. After spinning down cell lysate for analysis, the proteins were run on using gel electrophoresis with a NuPAGE 4-12% gradient Bis-Tris polyacrylamide gel and MOPS running buffer. A nitrocellulose membrane (Life Technologies Novex Cellulose Membrane) of .45 μm pore size was used to transfer the electrophoretic protein isolates within the western blotting chamber. The membrane was incubated in 2% blocking solution (GE Healthcare UK Limited, RPN2125V, Little Chalfont, Buckinghamshire, UK) with PBS-T for one hour at room temperature. The membrane was then incubated in the appropriate primary antibodies in PBS with 2% BSA and .1% sodium azide overnight at 4 °C. The following day, the membrane was washed three times with PBS-T and subsequently incubated in the corresponding enzyme-conjugated secondary antibodies to the primary antibodies applied (Vigodner *et al* 2016). The membranes were than washed thoroughly with PBS before adding chemiluminescence substrate and imaging.

In the apoptotic western blot, a negative control was established by applying DMSO to all cells and establishing duplicate rows of cells in the gel with DMSO solvent only and no GA treatment. A positive control was established by adding 10 uL of etoposide (2043S) from Cell Signaling Technology to one row. Etoposide, a cancer chemotherapeutic agent induces

proteolytic and apoptotic activity by signaling caspases and other proteases to degrade cell proteins. Therefore, applying it to a lane of sertoli cells demonstrates the appearance of definitive apoptosis in sertoli cells to provide a comparison by which to measure the apoptotic actions of GA on the cells. The successive samples of cells run on the gel were treated with increasing concentrations of GA, ranging from 10 to 25 to 50 μM, in duplicates, to ensure reliability.

The primary antibodies applied to the membrane included β-actin as a positive control, SUMO 2-3 to control for SUMO protein identification, and anti-parp as an apoptotic marker. Monoclonal β-actin antibodies are commonly used as a loading control in western blotting as actin is an essential component of muscle contraction and is expressed universally in all kinds of cells (Johnson 2012, Johnson 2013). Since studies have shown (Dittmer and Dittmer 2006) that incubating cells in beta actin antibody at higher protein loads or for longer periods of time lead to unreliable results for analysis, we incubated the membrane in 1:500 diluted actin antibody for just one hour. The anti-β-actin antibody from Santa Cruz (sc-1615), derived from goat, was diluted by a 1:1000 ratio before adding to the membrane. Another primary antibody was applied to the membrane to stain for SUMO 2/3 to detect change in SUMO protein content within the batches of treated sertoli cells. The anti-SUMO 2/3 antibody from Abcam (ab3742) derived from rabbit, was diluted by a 1:500 ratio before being added to the membrane. The final primary antibody added to the membrane was anti-parp. Parp describes a large family of nuclear proteins which play important roles in DNA repair, transcription, vasoconstriction, astrocyte and microglial function, as well as memory and aging. Parp-1, the most common and abundant of the family has been found to play a crucial role in cellular homeostasis by repairing DNA, and mediating cell signaling and

survival. Parp-1 can be cleaved by a number of suicide proteases, such as caspases, calpains and matrix metallopeptidases (MMPs) and therefore signal different forms of cell death once broken down into fragmented units. Since fragmented parp is a hallmark of apoptosis, we used a primary antibody that marks cleaved parp, signifying the activity of proteases in the cell breaking down its proteins for cell degradation and death, represented by a band at 90 kD on the membrane (Chaitanya *et al*, 2010). The anti-PARP antibody, from Cell Signaling Technology (9542s), derived from rabbit was diluted by 1:1000 before being added to the membrane.

In the proliferation western blot, DMSO was treated cells as a control yielding no expected changes due to added inhibitors alongside cells treated with GA. Therefore, all cells were cultured in DMSO and two duplicate wells of cells were treated with DMSO only and no GA as a negative control. The experiment was first run for a 3-hour period in which the treated cells were applied a 100 μM GA solution. A second experiment employed a 24-hour inhibitor incubation period and tested the effects of GA when applied to cells in concentrations of 75 and 90 μM, separately.

In this experiment, an anti-tubulin primary antibody served as a control to mark the presence of building blocks of microtubules, which are expressed across cell types. The mouse-derived anti-tubulin antibody was purchased from Abcam (ab7291-100) and diluted by a ratio of 1:10000. A rabbit-derived anti-SUMO 2/3 antibody purchased from Abcam (ab3742), diluted by a ratio of 1:500 was applied here to indicate presence of SUMO protein after GA treatment . An anti-PCNA antibody was then applied in order to determine whether GA impaired proliferation. PCNA, or proliferating cell nuclear antigen is a responsible factor for DNA replication and repair. It is expressed during the S phase or DNA synthesis stage of

the cell cycle and serves as a cofactor for DNA polymerase (Johnson 2012). Therefore, PCNA was used as a marker for proliferation, as differences in the detection of this protein could determine whether sumoylation inhibition could play a role in the proliferation of sertoli cells. The anti-PCNA antibody purchased from abcam (ab29) and derived from mouse was diluted to by a 1:1000 ratio and added to the membrane for primary antibody incubation.

After applying primary antibodies in both western blot experiments, secondary antibodies were added that were specific to detectors for the proteins of animals from which the primary antibodies were derived. Membranes were incubated in appropriate secondary antibodies for 1 hour at room temperature and then thoroughly washed with PBS-T. The secondary antibodies were enzyme conjugated and therefore produced a pigmented image on the membrane when activated by the chemiluminescent substrate and photographed by a CCD imaging device which can detect chemiluminescence. Membranes were viewed and analyzed under the Universal Hood II and Quantity one Software (Bio-Rad Laboratories, Hercules, CA). All experiments were performed in triplicates and DMSO was used continually as cell media.

V. Results

A. Viability Analysis

In the first experiment, sertoli cell lines were initially treated with GA in increasing concentrations and assessed for cell viability via the WST-1 assay. As presented in **figure 3**, while cells incubated in common cryopreservant DMSO shared constant absorbance values

in cells in the first column, cells treated with GA demonstrated a major decrease in cell viability beginning at 50 µM of GA. Control cells and DMSO treated cells were measured for an absorbance of about 3.7. However, cells treated with 50 µM of GA absorbed about 2.3. The next concentration of GA applied was 100 µM, only 50 µM greater than the first increment, yet the change in absorbance showed a greater than two times decrease in absorbance, at about .6. The next two sample rows which were treated with GA concentrations of 125 and 150 µM demonstrated stable absorbance values in accordance to that of the 100 µM treated cell line. Absorbance did not further decrease from there but rather remained constant.

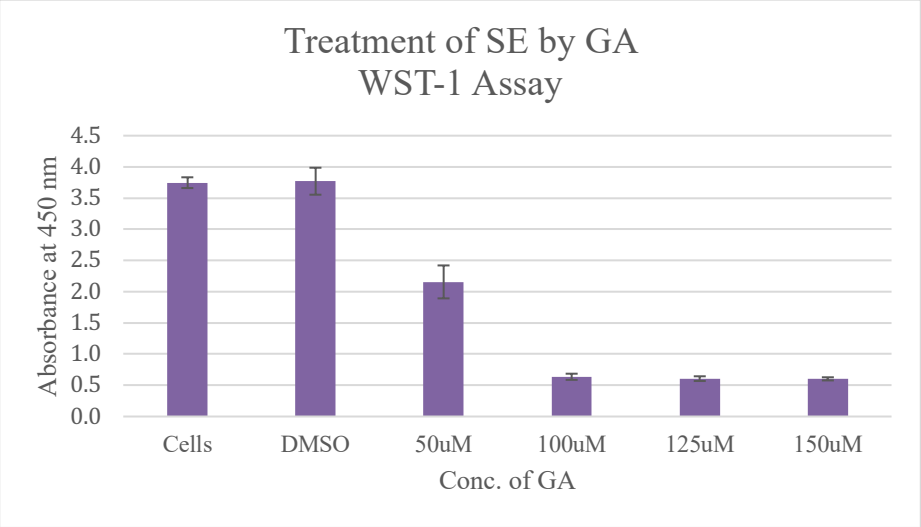


Figure 3.
WST-1 assay demonstrated that increasing concentration of GA caused a decrease in spectrophotometric absorbance. The addition of GA in columns 3, 4, 5, and 6 highlighted the decrease in cell viability that resulted from the inhibition of sumoylation in the sertoli cell lines. Cells with 50 uM GA absorbed almost half of the light that control cells did. Cells treated with 100,125,and 150 uM GA absorbed even less than half of the light compared to the 50 uM GA treated cells.

B. Apoptotic Study

Once the viability assay definitively confirmed the promotion of cell death by the inhibition of SUMO proteins in sertoli cells, we then sought to determine what specific mode of cell death is triggered in the absence of post-translational sumoylation.

While cell death occasionally occurs pathologically via necrosis or is stress-induced, or physiologically via senescent death, apoptosis has been identified as a systematic programmed shutting down of cells that are no longer useful or functioning properly. The process begins with the condensation and degradation of cellular materials and is followed by the phagocytosis and lysis of those particles (Kerr *et al*, 1972). Apoptosis is often a necessary and desired cell process in order to delete aberrant biological materials, regenerate renewable tissues and plays a major role in structural formation during embryonic development. This method of cell death eliminates the effects of methods like necrosis which may kill useful cells, trigger inflammation and leave remaining scar (Liu *et al* 2018).

When apoptosis is studied *in vitro*, it is important to note that a cell line on its own will never undergo apoptosis unless directly triggered. These cells designed for laboratory experimentation have been cultured to continually multiply and resist cell death in order to remain healthy and usable for experiments (Liu *et al* 2018). Therefore, an apoptosis assay performed on a cell line can specifically detect cellular shut down due to experimental conditions and treatments to the cell culture.

Along with the β-actin control and SUMO 2/3 antibodies which marked basic cellular materials to confirm proper sample loading and presence or dissipation of intact SUMO proteins, respectively, the PARP antibody was specifically chosen to identify signs of

apoptosis in GA treated sertoli cells. PARP is a known marker of apoptosis, as when it gets cleaved, it signifies that cellular proteases have been activated, as described above.

The results of the Western Blot presented in **figure 4** showed relatively equal loading of β-actin across the wells. While SUMO 2/3 was marked equally between the control DMSO and the low concentrations of 10 and 25 μM of GA lanes, in the 50 μM GA lane, SUMO staining was visibly decreased. Furthermore, the 50 μM sample presented visible markings at 90 kD, indicating the cleaving of PARP in these cells. The marking was not present at the same distance on the other lanes in the gel, both at GA concentrations of 10 and 25 μM as well as in the DMSO control sample. The etoposide treated cells, however, acted as a positive apoptosis control and demonstrated the cleaving of PARP in the first lane.

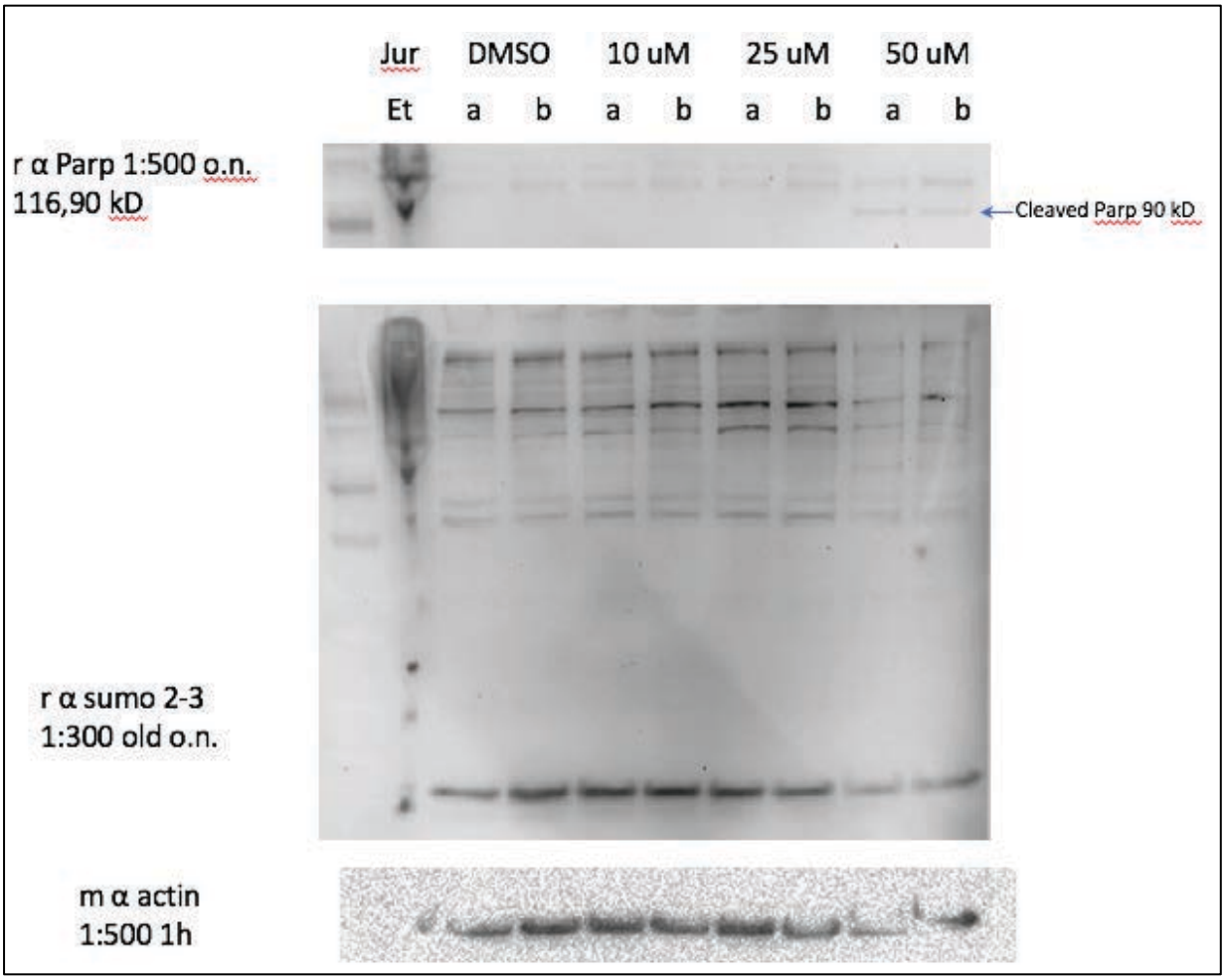


Figure 4.
The β-actin row demonstrates equal loading. SUMO 2-3 marker presents equal SUMO content across control and low concentration-GA-treated sertoli cells, however SUMO content appears lessened in the 50 μM GA sample. PARP cleavage distance identified in 50 μM GA lane only, as well, mirroring the cleaving point visible in the etoposide treated lane.

C. Proliferation Study

In the second western blot experiment performed, the proliferative effects of sumoylation inhibition were studied. In addition to the tubulin loading control and the SUMO 2/3 protein marker, anti-PCNA was used to detect the presence of this protein which aids cellular proliferation. PCNA, or proliferating cell nuclear antigen is a non-histone protein that

plays an essential role in DNA replication and synthesis. While it is known to be in lower concentration in some deteriorating cells, it marks an independent measure of cell proliferation since it is present in high levels during the G1 and S stages in the cell cycle, longer stages in which cells are constantly proliferating (Bologna-Molina *et al* 2013).

Sertoli cells in particular need to continuously proliferate through hormonal replenishment in order to support spermatogenesis through its many roles. Though spermatogenesis has mainly been believed to function mostly around the time of puberty, it has been posited that the process is repeated numerous other times during an organism's lifetime (Holstein *et al,* 2003). Sertoli cells must be proliferated and differentiated in order to allow the process to continue successfully. Thus, it was important to investigate whether the ability of sertoli cells to proliferate would be affected by the inhibition of sumoylation. It constitutes a different measure than the simple shut down of some of those cells and therefore needed to be independently studied.

As presented in **figure 5**, the tubulin control provided relative equal markers across the controlled and treated cells, as an indication that cells were properly loaded in each well. The SUMO protein detection also appeared mostly constant across each lane, with the exception of the cells treated with 100 μM GA for 3 hours on the left side of the figure. The PCNA marker was visibly constant with little deviation across each of the wells, whether controlled or GA treated. The results were uniform between the samples incubated with antibodies for 3 hours and those that were incubated for 24 hours.

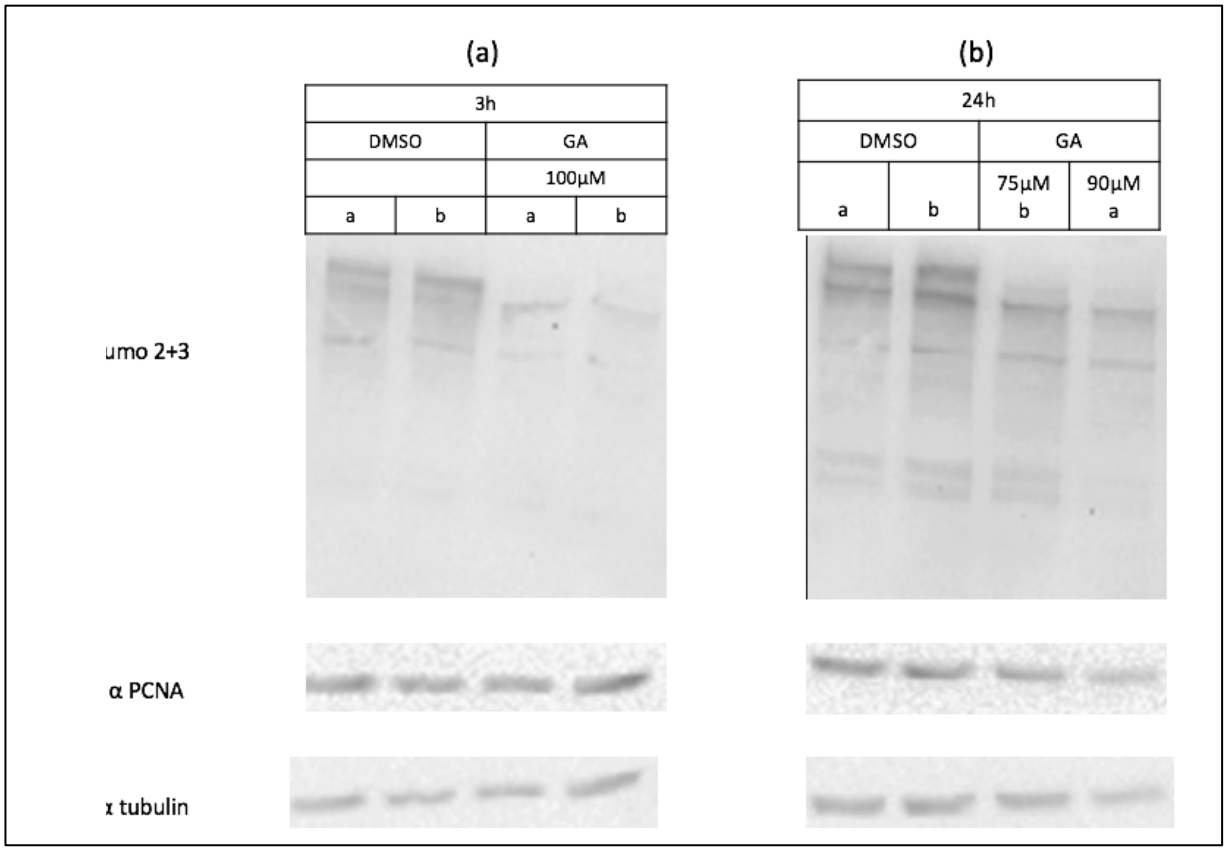


Figure 5.
(a)A control set of sertolicells in DMSO and in 100 µM each incubated in antibody for 3 hours show no significant differences in loading control tubulin markers, SUMO protein presence, as well as PCNA cell proliferation marker
(b) On a membrane incubated for 24 hours, samples of SCs in DMSO as well as those treated in both 75 and 90 µM of GA presented no significant difference in marked tubulin loading control, SUMO protein expression and PCNA proliferation markers.

VI. Discussion

We sought to investigate and address three important questions through the experiments in this particular study. The Vigodner lab has spent many years researching the role that sumoylation plays in the various stages of spermatogenesis which contribute male infertility. Sumoylation is a more recently discovered and not fully understood post-

translational modification that may have powerful effects on cell mortality and the success of the processes which it undergoes during its lifetime.

In a number of research studies, including some conducted our lab, the common SUMO 1 and SUMO 2/3 proteins have been detected and analyzed within spermatogonia at earlier stages of development, within sex chromosomes, as well as in various areas of the later developed spermatids, and even within the nuclei of somatic testicular cells (Vigodner *et al* 2016). This last finding has led us to further investigate the less understood importance of SUMO proteins within sertoli cells, a unique but crucial type of somatic cell present in the testes which mediates the development of spermatids throughout the process of spermatogenesis.

The first question we sought to address was whether sumoylation is a process essential for the survival of a sertoli cell. Using a purchased sertoli cell line, we cultured a batch of sertoli cells and treated them with varying concentrations of GA, a specific inhibitor of SUMO protein actions. We performed a WST-1 assay by adding the appropriate reagents to these treated cell plates and measured the absorbance according to the pigmentation of each treated sample. Since the WST-1 is a tetrazolium salt, when it was added to the treated cells, it got easily reduced to a purple dye, formazan as long as reducing agents were present in the cells. The absorbance and therefore the concentration (directly proportional through Beer's Law) of the pigmentation visible and detectable by the spectrophotometer after the experiment was therefore higher in cells that were treated with lower concentrations of GA.

Compared to the measured 3.7 level of absorbance in the untreated and DMSO control cells, the sample of cells treated with 50 μM GA decreased in absorbance by a factor of about .7 and absorbed light a level of 2.2 when measured at a wavelength of 450 nm. Cells

treated with GA concentrations of 100, 125 and 150 μM demonstrated an even steeper decline in absorbance, with absorbance levels of about .6, which is less than a sixth of the absorbance value of sertoli cells with no treatment. Interestingly, there were no further changes in absorbance between the samples treated with 100, 125 and 150 μM of GA, suggesting that any cell line treated with GA of a concentration above 100 μM would absorb light in the same way.

The clear decrease in absorbance with the addition of GA inhibitors to the sertoli cells indicated that inhibition of sumoylation indeed inhibits the viability of sertoli cells. As described above, the decrease in absorbed pigmentation resulted from degraded cells no longer possessing the reducing agent electron acceptors available in metabolically active cells. The further drop in absorbance between cells with 50 μM of GA and 100, 125 and 150 μM of GA suggested that sumoylation inhibition may impact cell viability in a dose dependent manner. Further studies would need to verify the validity of those differences and additionally investigate whether clinical treatments to restore sumoylation capabilities would need to be moderated by dose as well.

Once we were certain that sumoylation was essential for sertoli cell viability, we faced the next task to determine the particular mode of cell death sertoli cells undergo when unable to perform sumoylation. There are numerous routes by which cells can be shut down, as discussed above, and each may be targeted by different therapeutics accordingly. Thus, we performed a western blot analysis to test whether apoptosis, or programmed cell death was the particular method employed by sertoli cells to shut down when its SUMO proteins are inhibited.

The western blot imaging presented in **figure 2** suggested that SUMO proteins began to be less detected in the SCs treated with the highest concentration of GA used, 50 μM, compared to the other concentrations of GA and the negative DMSO control. This indicated that a minimum of 50 μM of inhibitor was required to impair SUMO function in the cell.

This sample of cells treated with the highest GA concentration proved to be the point at which apoptosis was activated in the Sertoli cells, as also indicated by the marker of parp cleavage at 90 kD, which signified that cellular proteases had begun to degrade proteins within the cell and position them for phagocytosis by other cells, as the process of apoptosis describes (Kerr 1972). The clear parp cleavage markings on the membrane allowed us to conclude that apoptosis was certainly the mode of death that Sertoli cells employed when sumoylation was inhibited due to sufficient concentration of GA.

Once we knew that the inhibition of sumoylation impaired the viability of sertoli cells via apoptosis, we wanted to test whether sumoylation inhibition would also impair sertoli cells from proliferating. Not only is proliferation of spermatogonia an important step in the process of spermatogenesis, but the replication and replenishment of sertoli cells is also crucial for the continued support of developing spermatogonia in the testes. Thus, we ran another western blot, but this time tested for a proliferation marker.

The results presented in **figure 3** indicate no change in sertoli cell proliferation after varying concentrations of GA had been added to cells. We ran one gel in the experiment but incubated parts of the membrane in the primary antibody for different amounts of time in order to compare changes when more time was provided to allow for antibody-antigen binding.

The results presented that whether the membrane was incubated for 3 hours or 24 hours, or in 75, 90, or 100 μM GA, the PCNA marker remained uniform and unchanged, showing that proliferation is not impaired by sumoylation inhibition. The marker for SUMO ⅔ protein appears dissipated in the 100 μM GA sample treated for 3 hours, confirming that this sample underwent the greatest sumoylation inhibition. The SUMO marker in the other GA treated samples did not display visible change as compared to the DMSO control. From this last experiment, we were able to rule out proliferation as a significant change that occurs with the inhibition of sumoylation in sertoli cells. Based on our data, the effects of GA were conserved to cell death via apoptosis but did not change the degree to which sertoli cells multiply.

VII. Conclusion

Sumoylation has been increasingly studied in recent years, and particularly in our lab's research, we have explored its significant post-translational modifications within cells of the male reproductive system. While we have focused mainly on the presence of SUMO proteins in the spermatogonia of the testes which develop into functional, motile spermatids, in this research project, we sought to determine the effects of sumoylation in a somatic cell found in the testes which provides essential support to the germ cells of the testes, sertoli cells.

Based on the experiments we performed, it was concluded that the inhibition of sumoylation in these cells significantly reduced the viability of the cells. Upon further investigation, we confirmed that sertoli cells lost their viability via apoptosis when they were

lacking SUMO proteins to perform this necessary cellular modification. Our proliferation study however did not provide evidence that proliferation was impaired by sumoylation inhibition.

These results have allowed us to focus future research questions toward meaningful therapeutic conclusions. In future studies we must first determine how exactly apoptosis is activated upon sumoylation inhibition. There are numerous cellular pathways that we have explored in this paper and in others that play important roles in the functions of sertoli cells and in sumoylation modifications. By determining which pathways and cellular proteins are directly involved in sertoli cell apoptosis, we will be able to more effectively target those areas with appropriate treatments. In addition to how exactly apoptosis is activated, we must also explore which particular proteins within sertoli cells are being sumoylated successfully, and therefore which are impaired sumoylation is inhibited. For example, we must investigate which proteins are involved in activating parp when apoptosis is activated.

Furthermore, once we identify these specific target proteins and pathways, we must then go beyond mouse-derived cell line experiments and verify the results of our research by running the same experiments on primary human sertoli cells for reliable, relevant data that can provide real clinical applications. If these stages prove successful, we will conduct final research studies *in vivo* via genetic inactivation in mice.

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