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The Effects of Insulin and Glutathione on In Vitro Spermatogenic Cyst Maturation

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THE EFFECTS OF INSULIN AND GLUTATHIONE ON *IN VITRO* SPERMATOGENIC CYST MATURATION

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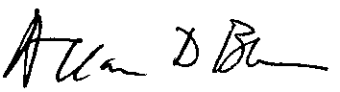

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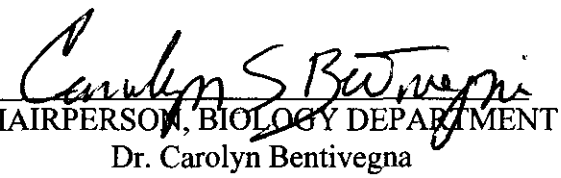

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ABSTRACT

Spermatogenesis is a series of complex processes that leads to the development of sperm cells. It involves both mitotic and meiotic cell divisions followed by dramatic cytoskeletal reorganizations and cell growth . Previously our lab developed a culture system for *in vitro* study of spermatogenesis using isolated spermatogenic cysts from the testes of *D. pseudoobscura* (Njogu *et al.* 2010). The use of *D. pseudoobscura* is advantageous compared to the model organism *D. melanogaster* because: 1) survival of cysts to the elongated and motile form is easily achieved and repeatable and 2) minimal media is required for the growth of the spermatogenic cysts.

Insulin-like peptides (ILPs) are known to play a role in the progression of *Drosophila* spermatogenesis *in vivo*. ILPs are involved in the signaling pathway responsible for the maintenance of spermatogenic stem cells in the testis stem cell niche. Specifically, ILPs are important for the growth of spermatogonia and maturation of these cells into primary spermatocytes (Ueishi *et al.* 2009). Glutathione (GSH) is a known antioxidant that plays an important role in both plant and animal culture systems (Meloni *et al.* 2003). The purpose of the current work was to determine the effects of exogenous insulin and glutathione on *Drosophila* spermatogenic cyst growth *in vitro*. Our results indicate that the addition of glutathione alone to our culture system has the strongest effect on spermatogenic cysts survival *in vitro*. Additionally, nuclear fluorescence staining with acridine orange and DAPI (diamidino-2-phenylindole) indicates that post-meiotic nuclear transformation occurs normally in our cultured cysts.

INTRODUCTION

Overview

Over the past century, *Drosophila melanogaster* has been used as a model organism for genetic, developmental, and cell biological studies. These flies are an attractive system of study because of their short generation time, the availability of fully sequenced genome, and the accessibility to genetic manipulation. In addition, they can be cheaply maintained in the laboratory. With these advantages, *Drosophila melanogaster* is currently one of the most important model organisms utilized in biological investigations. *Drosophila melanogaster* has been the prevalent species in spermatogenesis studies *in vitro* and *in vivo* (White-Cooper 2010; Fuller *et al.* 1993; Gregory *et al.* 2008; Cenci *et al.* 1994; Ueishi *et al.* 2009).

Spermatogenesis in *Drosophila*

Sperm production continues throughout adulthood in most male animals. In both flies and mammals, regeneration of stem cells provides a continuous supply of precursor sperm cells, so spermatogenesis is maintained via a stem cell system (White-Cooper 2009).

Spermatogenesis in *Drosophila* proceeds within paired blind-ended tubular or ellipsoid testes made of muscle and pigmented cells. It begins with the division of diploid germline stem cells (GSCs) at the apical end of the testes, which progress to become individual highly specialized, motile sperm (Fig 1). The germinal proliferation center, otherwise known as the hub, is located apical end of the testes. It consists of three types

of cells: GSCs, somatic stem cells, and hub cells (Hardy *et al.* 1979). The mitotic division of a GSC results in a gonialblast committed to differentiation and a GSC that remains in contact with the hub. In *Drosophila*, the somatic stem cells, or cyst progenitor cells (CPCs), produce the cyst cells that accompany the differentiated spermatogonia throughout spermatogenesis (Fuller *et al.* 1993). To maintain their stem cell characteristics both stem cell populations, GSCs and CPCs, are arranged in a rosette around the hub cells, a population of approximately twenty post-mitotic cells also at the apical end of the testes (Hardy *et al.* 1979). The hub cells constitute a signaling center responsible for maintaining normal stem cells (White-Cooper 2010).

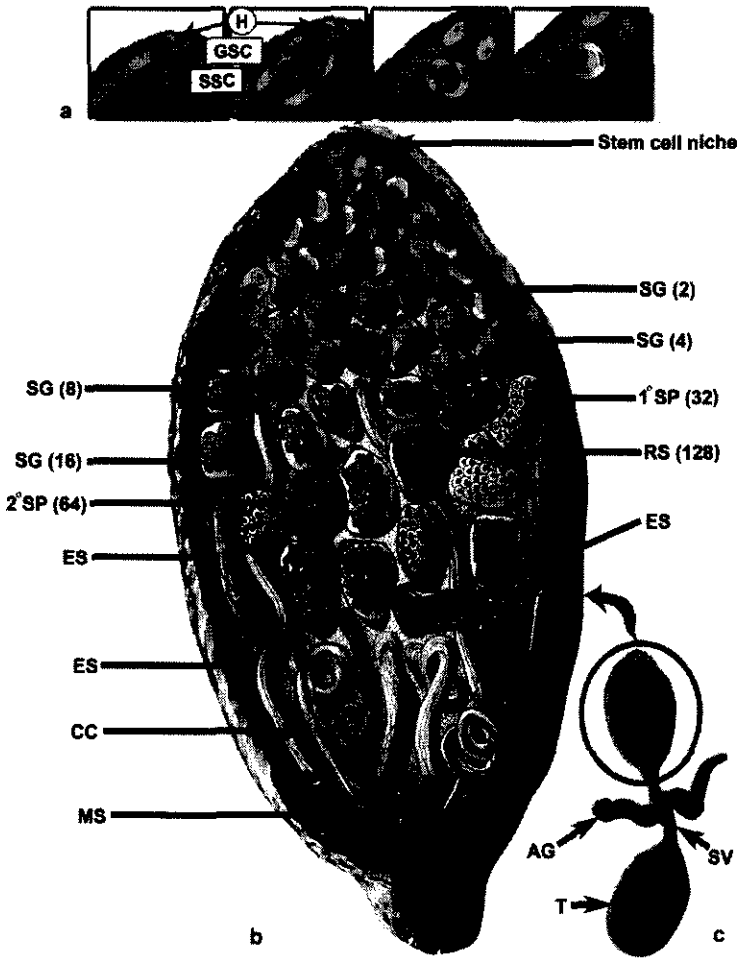

Fig. 1. Diagrammatic representation of spermatogenesis in *D. pseudoobscura*.
(a) Enlargement of the stem cell niche. The panel shows the progression of the encapsulation of a gonialblast, from left to right. H = hub cells; GSC = germline stem cell; SSC = somatic stem cell (cyst progenitor cell). (b) Enlargement, cut-away view of the testes showing the stages of cyst development. SG = spermatogonia; 1° SP = primary spermatocyte; 2° SP = secondary spermatocytes; RS = round spermatids; ES = elongating spermatids; MS = mature spermatozoa; CC = coiling cyst. (c) Illustration testes, with the accessory glands (AG) attached. Mature sperm are stored in the seminal vesicles (SV) until mating. T = testis. Illustration after Njogu *et al.* 2010, Cell and Tissue Research, in press.

Each mitotic division of a cyst progenitor cell (CPC) produces a CPC and produces a cyst cell. Two cyst cells, analogous to the Sertoli cells of the mammalian testis, encapsulate each gonialblast. The cyst cells terminally differentiate by completing mitosis while the encapsulated gonialblast continues to differentiate. The spermatogenesis process continues within the cysts. The gonialblast undergoes a series of incomplete mitotic divisions to produce spermatogonia, followed by a growth phase that gives rise to primary spermatocytes in syncytium within the cyst. Cytokinesis is incomplete during these divisions, which leaves the spermatogonial cells connected by stable intercellular bridges called ring canals (Fuller 1998). Intercellular cytoplasmic bridges are important to maintain developmental synchrony among the spermatogonial cells. In *D. melanogaster*, there are four mitotic divisions that result in a cyst with 16 primary spermatocytes, while in *D. pseudoobscura* there are five mitotic divisions that give rise to cysts with 32 primary spermatocytes (Scharer *et al.* 2008).

Many of the processes of spermatogenesis in mammals are conserved in *Drosophila.* As in mammalian spermatogenesis, the primary spermatocyte stage is characterized by incomplete cytokinesis, extensive cell growth, and an increase in gene expression (White-Cooper 2009). The primary spermatocytes rapidly progress through pre-meiotic S-phase, and then enter an extended G2 cell cycle phase in which they grow up to 25 times in volume. The conventional stages of meiotic prophase are not visible in *Drosophila* males, which indicates that meiotic recombination of chromosomes does not occur in the cells (Fuller 1998). At the end of the primary spermatocyte stage gene expression ceases, and the cells progress through two meiotic divisions which yield a cyst

with interconnected haploid spermatids. In *D. pseudoobscura*, meiosis yields 128 haploid spermatids that progress towards the sperm maturation process termed spermiogenesis (Fig. 1).

As in mammals, spermiogenesis in *Drosophila* occurs by a series of drastic morphological changes. The spermatids grow a long flagellum containing an axoneme, a microtubule-based organelle, for motility. In addition, change in nuclear structure and DNA condensation occurs (Fuller 1998). During elongation, sperm nuclei transform from a spherical shape to an elongated "needle-shape" that is condensed. As in mammalian spermatogenesis, a switch in structure from nucelosomal-based to protamine-based chromatin takes place in *Drosophila*. It is well established that in mammals nucleosomal confirmation is lost after meiosis due to multiple modifications of histones proteins (Clermont *et al.* 1993). Rathke *et al.* (2007) reported similar modifications of histones in *Drosophila* prior to their degradation, and the presence of protamines around sperm nucleus. Histones are removed from chromosomes of spermatids and replaced initially by transition proteins, then protamines. This process results in a highly condensed state of the chromatin, which is essential for the transmission of the male genome to the oocyte (Rathke *et al.* 2007; Oliva 2006). Previous studies have reported that a knockout of only one protamine allele, P1 or P2, in mice was sufficient to cause infertility (Cho *et al.* 2001).

Fully elongated cysts in *Drosophila* proceed to the next phase of spermiogenesis known as individualization, where each sperm cell becomes invested in an individual plasma membrane. This process involves the formation of an individualization complex,

which is dependent on apoptotic pathway activation. According to the findings of Arama *et al.* (2003) caspases are apoptotic proteins important during individualization in *Drosophila melanogaster*. Caspase activity was inhibited in both cultured testes and *in vivo*, which caused the individualization process to become aberrant. The individualization complex is a coordinated array of actin cones which move synchronously along the cyst, with each spermatid being individualized by a single investment cone. This cytoskeletal-membrane complex assembles at the nuclear end of the cyst and progresses to the tails investing each spermatid in its own plasma membrane, while simultaneously expelling excess syncytial cytoplasm and organelles (Tokuyasu *et al.* 1972; Farizio *et al.* 1998). Movement of the complex along the spermatogenic cyst is observed by the presence of an enlarged region known as the cystic bulge. Noguchi and Miller (2003) reported that actin polymerization is responsible for cystic bulge movement during individualization. The cystic bulge consists of accumulated cytoplasmic material and degrading organelles. The bulge is detached from the mature individualized cyst when it reaches the tail end, where it is referred to as a waste bag (Tokuyasu *et al.* 1972). In the presence of caspase inhibitors the individualization complex remains in the vicinity of the sperm nuclei, which gives indicate a defect in its translocation along the cyst (Arama *et al.* 2003). Individualization of spermatids is followed by the coiling of the mature spermatids towards the basal end of the testes, where mature sperm are then transported into the seminal vesicle for storage (Tokuyasu *et al.* 1972).

Previously, Cross and Sang (1978) used an *in vitro* culture system to compare the development potential of cell types of normal and mutant embryos in a condition where

they are relatively isolated. Since then, there have been several attempts to culture *Drosophila* germline cells *in vitro*. *In vitro* culture of isolated cysts from *D. hydei* has been achieved by Fowler (1973), Fowler and Johannison (1976) and Liebrich (1981 and 1982). Fowler (1973) maintained the testes from pupae of *D. hydei* in culture. In that study, there were minimal degenerative changes in primary spermatocytes after two days in culture, which gave evidence that the testes were being maintained normally. However, after two days development of late spermatids and the process of spermiogenesis was not observed *in vitro*. Liebrich (1981) reported elongated and coiled cysts in culture; however the process of individualization was not seen as in *D. melanogaster*. He also reported that *D. hydei* cysts isolated when their spermatocytes were in prophase I differentiated for 2-3 days at 22°C *in vitro*. However, at lower temperatures, differentiation was prolonged to approximately 5 days. He further reported that spermatogonial cysts were difficult to cultivate for longer than 3 days. Cross and Shellengarger (1979) successfully cultured isolated cysts *in vitro* from wild-type testes of *D. melanogaster*. The development of late cysts from meiosis to the coiling stage was observed in culture. However, motility was never observed, cysts only elongated to 1/3 their normal length, and maturation success rate was low. In more recent studies, *in vitro* culture of *D. melanogaster* adult (Noguchi and Miller 2003) and larval testes (Kawamoto *et al.* 2008) has been successfully achieved. Adult cyst culture investigated the mechanism of sperm individualization, while in larval cyst culture sperm motility was consistently achieved *in vitro*.

In our previous study, we successfully isolated and cultured spermatogenic cysts from the pupal-stage testes of *D. pseudoobscura* (Njogu *et al.* 2010). The testes of *D.*

pseudoobscura can be easily distinguished in the later pupal stages due to intense pigmentation, and are easily handled due to their ellipsoid morphology (Fig. 2). The survival of cysts to the elongated and motile form was consistently achieved *in vitro*. However, the degeneration of early spermatogenic cysts as well as primary spermatocytes was frequently observed in the culture system. Previously, Niki *et al.* (2006) established an *in vitro* system to analyze factors that regulate the division and differentiation of GSCs in *Drosophila* ovaries. They tested various culture conditions by adding insulin, GSH, and fly extract. Kawamoto *et al.* (2008) reported the differentiation of primary spermatocytes and dissociated spermatogonia into motile spermatids *in vitro*. Exogenous insulin and glutathione (GSH) was incorporated in their culture system. Insulin is known for its role in stimulating the uptake of glucose into cells. However it can also act as a mitogen for various cell types in culture (Fernandez *et al.* 1995). An insulin receptor and its signaling cascade are well conserved in *Drosophila* (Fernandez *et al.* 1995). *Drosophila* insulin-like peptides are known to promote the proliferation and growth of somatic cells, and the division of GSCs in females (Ueishi *et al.* 2009). In mammals GSH is involved in the protection of both males and female gametes against oxidative damage (Luberda 2005).

Fig. 2. *D. pseudoobscura* pupae and testes. Isolated testes indicated by the white arrows. Pigmented testes can be observed through the abdominal wall of the fly (black arrow).

The purpose of our current study was to: 1) study the effects of exogenous insulin and glutathione on spermatogenic cyst maturation *in vitro* and, 2) compare the integrity of the chromatin in cultured sperm to the chromatin of sperm cells *in vivo* using nuclear fluorescent staining techniques. In non-treated cultures as well as those treated with insulin only, glutathione only, and insulin + glutathione the survival of cysts to the elongated, motile form was consistently achieved. Quantitative analysis showed that spermatogenic cysts in cultures treated with glutathione only and insulin only out-performed the non-treated cultures after 96 hours *in vitro*, while spermatogenic cysts in cultures treated with insulin + glutathione performed comparable to those in non-treated cultures after 96 hours *in vitro*. Additionally, no difference in chromatin integrity was detected in cultured sperm nuclei compared to sperm nuclei produced *in vivo*.

MATERIALS AND METHODS

I. Fly Stock and Cultures.

Fly stocks were obtained from the University of California San Diego Drosophila Species Stock Center. All flies were cultured in our laboratory on Jazz Mix Drosophila medium (Fisher) at 25°C.

II. Culture Media

The culture media used was based on previous studies by Cross and Shellenbarger (1979). Powdered Shields and Sang M3 Insect medium without bicarbonate (Sigma-Aldrich, St. Louis MO), was reconstituted according to manufacturer's instructions, and supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin cocktail (Sigma-Aldrich) according to previous studies (Niki *et al.* 2006; Kawamoto *et al.* 2008). The serum was heat-inactivated at 50°C for 30 minutes. Treated cultures were supplemented with either 10 μg/ml insulin from bovine pancreas (Sigma-Aldrich; Freshney 1994.), 0.6 mg/ml glutathione (Sigma-Aldrich), or both insulin and GSH. Culture media was prepared with glutathione-only, insulin-only, and insulin + glutathione on the day of culture. Untreated control cultures were grown in supplemented medium that lacked insulin and glutathione.

III. *In vitro* culture of isolated cysts

Pupae were harvested five to seven days after pupation began. They were soaked in 70% alcohol for 15 minutes, and dissected in 1X phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Sigma-Aldrich) on a sterilized bench. After dissection, three testes were washed twice in 1X PBS and once in 50 µl supplemented culture media. The testes were then ruptured in supplemented culture media with forceps. The released cysts were dissociated by gently pipetting several times. The dissociated cysts were then transferred, using a pipette, into a sterile 24-well culture plate with fresh culture media under the laminar flow hood. Each well contained 950 µl of supplemented M3 media, and 50µl of cysts. The cysts were cultured at room temperature (22°C) without CO_2.

Cysts cells were monitored daily and images collected using an inverted, phase contrast Leica DMIL microscope equipped with a Leica DFC 300 CCD camera. After 48 hours in culture, 200µl of supplemented media was added to each culture system.

IV. Quantification of cyst growth

Cysts were quantified by manually counting viable cysts present at each stage of development over the course of 96 hours in culture. Normal cysts were defined as those that had normal morphology and lacked any kind of degeneration such as bursting of cysts. The cysts were categorized in developmental stages based on the number of cells per cyst, morphology, and size of the cysts. A total of three experiments was performed for glutathione-only, insulin-only, and insulin + glutathione cultures. A total of six

experiments was performed for non-treated cultures. The duration of manual counting of cysts for quantification for treated and non-treated cultures was approximately thirty minutes. This time was kept as short as possible to minimize the exposure of cysts to light. Data collected from experiments for each treatment condition was pooled and statistically analyzed using the software package Tableau and GraphPad.

V. Fluorescence staining of *in vitro* culture with isolated cysts with DAPI

For florescence staining with DAPI, the testes and isolated cysts were obtained as described in section III above. However instead of culturing isolated cysts in 24-well plates, cysts were cultured in Culturewell chambered cover-glasses (Electron Microscopy Sciences). Dissociated cysts were transferred, using a pipette, into a chamber with fresh culture media under the laminar flow hood. Each well on the cover slip contained 300 µl of supplemented M3 media, and 50 µl of cysts. After 72 hours in culture 3.5 µl of 1 mg/ml DAPI fluorescent nuclear stain (Invitrogen, USA) was added to the cover slip under the laminar flow hood, and incubated at room temperature for 10 mins. A 25 µl drop of the stained cysts from the chamber was added to a glass slide, and a single cover-slip was gently placed on top of the drop.

Fluorescence staining of cysts obtained from pupae (24-48 hrs before eclosion) with DAPI (Invitrogen) was also performed as a control. Three pupae were dissected in 1X phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Sigma-Aldrich) on a sterilized bench. After dissection, the three testes were washed twice in 1X PBS. The testes were ruptured in 100 µl of 1X PBS with forceps. The released cysts were stained

with 10 μg/ml of DAPI. The mixture was gently stirred with a pipette and incubated for 10 minutes. A cover-slip was gently placed on top of the drop.

Images were collected using an Olympus BX40 epifluorescence microscope and an inverted, phase contrast Leica DMIL microscope equipped with a Leica DFC 300 CCD camera.

VI. Fluorescence staining of *in vitro* culture with isolated cysts with acridine orange

For fluorescence staining with acridine orange, the testes and isolated cysts were obtained and cultured as described above for DAPI. After 72 hours in culture, 20 μl of the culture media with cysts and 5 μl of 100μg/ml acridine orange (Sigma-Aldrich) were transferred to a glass slide for a final concentration of 20 μg/ml. The mixture was gently stirred with a pipette, and a cover slip was gently placed on top of the drop.

Fluorescence staining of adult flies with acridine orange was also performed as a control. Three adult flies were dissected in 1X phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Sigma-Aldrich) on a sterilized bench. After dissection, the three testes were washed twice in 1X PBS. The testes were ruptured in 20 μl of 1X PBS with forceps. The released cysts were stained with 5μl of acridine orange (Sigma-Aldrich). The mixture was gently stirred with a pipette, and a cover-slip was gently placed on top of the drop.

Images were collected using Olympus Fluoview 1000 confocal laser scanning microscope (CLSM) and an Olympus BX40 epifluorescence microscope.

RESULTS

I. Culture of isolated of cysts

i. Glutathione only

Cysts from pupal testes were isolated and cultured with exogenous glutathione. This agent was used to investigate whether its anti-oxidant properties might rescue the degradation of early stage cysts noted in our previous study. Staging of cultured cysts was done using cyst size, number of germ cells within the cysts, the shape and size of the germ cell nucleus, as well as the general morphology of the cyst. When initially isolated from testes, cysts in advanced post-elongation stages of differentiation (individualization and coiling), were never observed. Qualitatively, both development and degradation of the cysts was apparent over four days in the cultures treated with GSH and non-treated cultures (Figs. 3d and 4d). Cyst maturation was observed after 24 hours in both cultures, indicated by the presence of the cystic bulge along a number of cyst cells and initial coiling of cysts (Figs. 3a and 4a). By day four in culture cysts were fully developed and motility was observed in treated and untreated cultures.

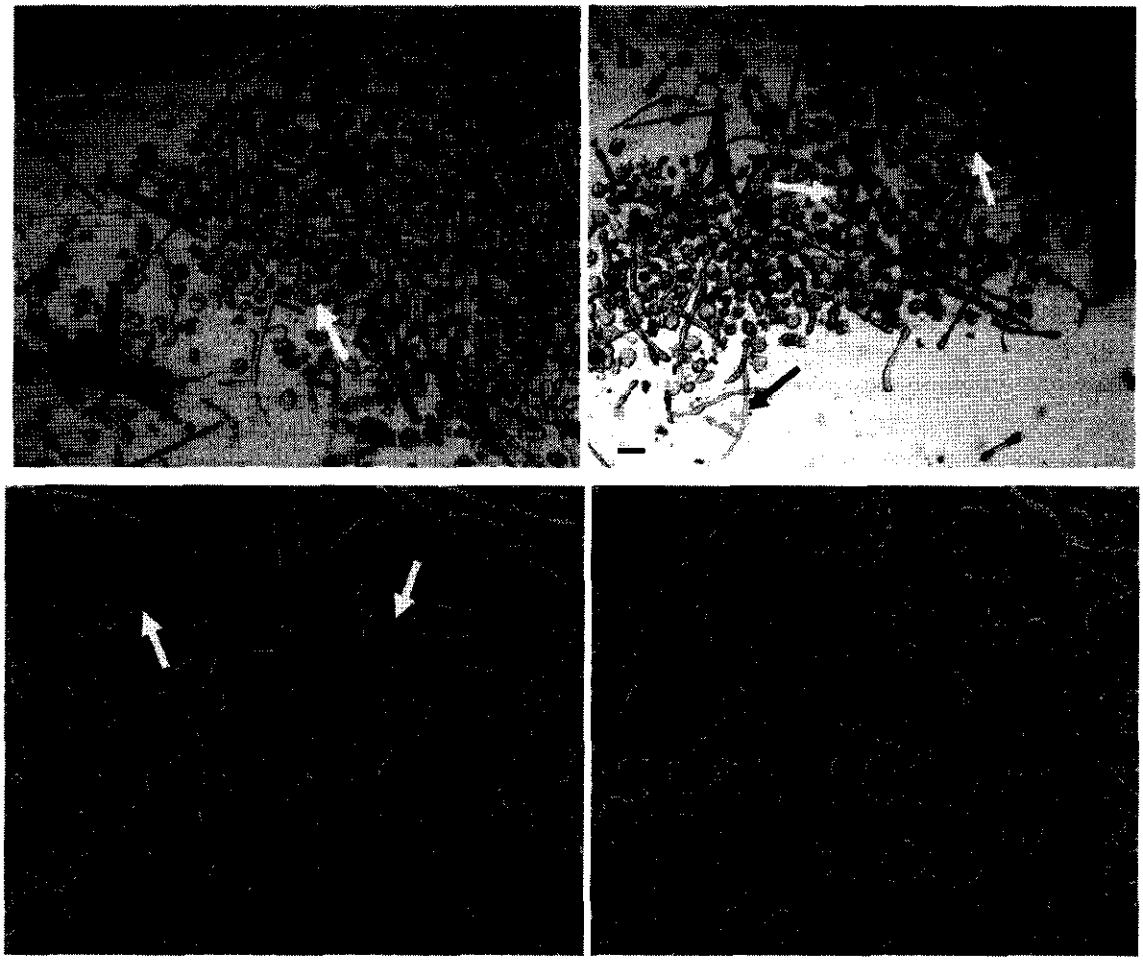

Figure 3. Isolated cysts after 24 hours and 96 hours in culture medium supplemented with GSH. (a) Cysts after 24 hours in culture, note the appearance of a coiled cyst (black arrow) and a cyst undergoing individualization (white arrow). (b) Cysts after 96 hours in culture. Note more elongating cysts have a cystic bulge, indicative of individualization (black arrows). In addition an increase in coiling cysts was apparent (white arrows). (c) Phase contrast micrograph of cysts after 24 hours in culture. Note the appearance of degenerating cysts (black arrows) as well as early elongating cysts (white arrows). (d) Phase contrast micrograph of isolated cysts after 96 hours in culture. Note most early cysts have degenerated. Fully coiled cyst was also apparent (black arrow). Scale bars = 100 μm in all panels.

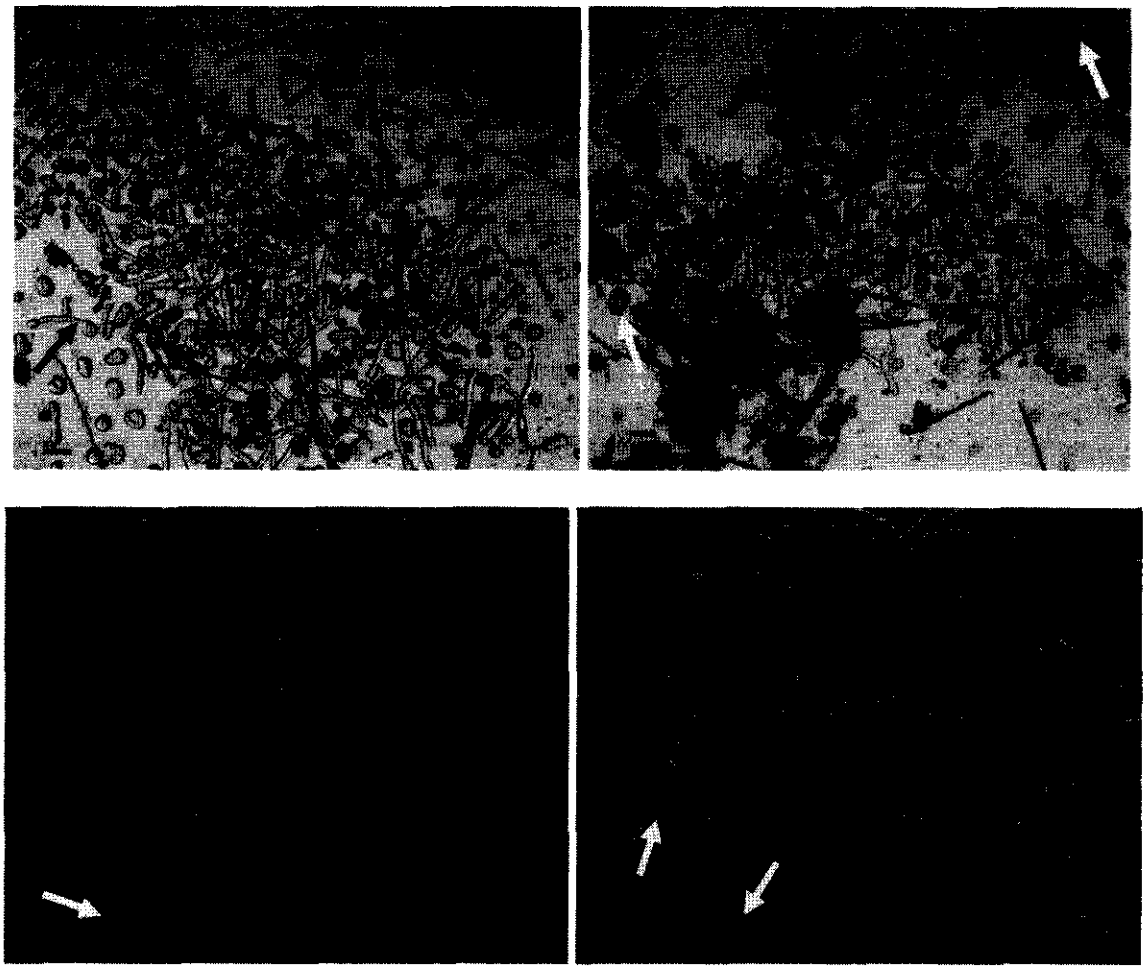

Figure 4. Isolated cysts at 24 hour and 96 hours in culture with no treatment. (a) Cysts after 24 hours in culture, note the appearance of initial coiling of cyst (black arrow). (b) Cysts after 96 hours in culture. White arrows indicate coiled cysts. In addition an increase in coiling cysts was apparent (black arrows). (c) Phase contrast micrograph of cysts after 24 hours in culture. Note the appearance of early elongating cysts (black arrow) as well as cyst beginning to coil (white arrow). (d) Phase contrast micrograph of isolated cysts after 96 hours in culture. Note most early cysts have degenerated. Cysts at the individualization stage (white arrows) and initial coiling stage (red arrow) were apparent. Scale bars = 100 μm in all panels.

In cultures treated with GSH, consistently after 24 hours, quantitative analysis showed a rapid increase in meiotic cysts and cysts at the elongation stage of development compared to non-treated cultures (Table 1). However, there was a significant decrease in the number of mitotic cysts, which include spermatogonia and primary spermatocytes (Tables 1 and 4). After 48 hours, the number of mitotic cysts continued to decrease but the number of meiotic and late stage cysts increased. Interestingly, after 48 hours the percentage of coiling cysts was significantly higher in the non-treated cultures as compared to the treated cultures (Figs. 4 and 11). This trend continued up to 96 hours.

By day four of culture, a higher percentage of early cysts was observed in cultures treated with GSH compared to non-treated cultures. Even though there was a significant decrease in mitotic cysts in both cultures, their survival rate was higher in the cultures treated with GSH over the non-treated cultures. A ratio of GSH performance on cyst survival in the cultures went from a factor of 1.78 at 48 hours to 1.96 by 96 hours, which further suggests GSH has a significant effect on pre-meiotic cyst survival over time (Fig. 10).

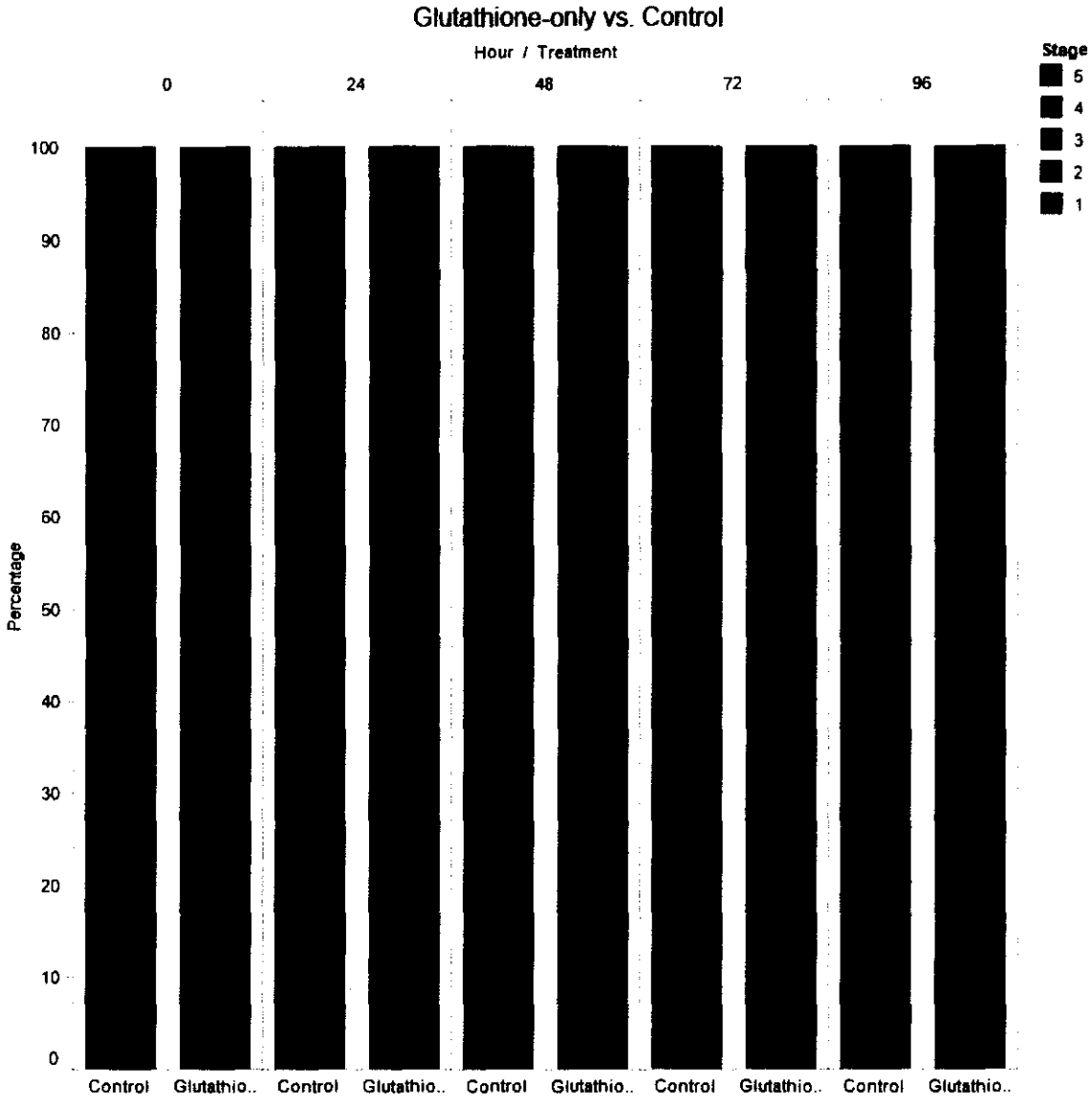

Figure 5. Percentage of viable cysts at different stages of development in cultures treated with GSH compared to non-treated over 96 hours. (1) Spermatogonia (in purple) (2) Primary spermatocytes (in dark blue) (3) Secondary spermatocytes/Round spermatids (in light blue) (4) Elongating and individualizing cysts (orange) (5) Coiling cysts (red). Data from a series of experiments were pooled.

ii. Insulin

Insulin was added independently to the cultures to investigate whether it can act to rescue the degradation of early stage cysts noted in our previous study. As mentioned previously, staging of cultured cysts was done using cyst size, number of germ cells within the cysts, the shape and size of the germ cell nucleus, as well as the general morphology of the cyst. As with GSH-only treated cultures, post-elongation development and early cyst degradation was apparent after 24 hours (Figs. 4 and 6).

After 24 hours, quantitative analysis showed an increase in meiotic cysts, and cysts at the elongation stage of development in cultures treated with insulin compared to non-treated cultures. In addition, there was a decrease in the number of primary spermatocytes (Table 2). After 48 hours the number of meiotic cysts in the treated cultures continued to increase, however in the non-treated cultures only a slight increase was observed. The non-treated cultures had a greater number of cysts at the coiling stage compared to treated cultures (Tables 2 and 4). Both cultures, treated and non-treated, had a comparable number of cysts at the coiling stage between 24 and 48 hours. However after 72 hours the non-treated cultures had 17.9% of coiled cysts and the treated cultures had 13.2% (Fig. 11). By day four, the non-treated cultures out-performed the treated cultures by 10% (Fig. 11). For the survival of mitotic cysts, the ratio of insulin performance in the treated cultures over the non-treated cultures went from a factor of 1.27 at 48 hours to 1.89 by 96 hours (Fig. 10). These results indicated that insulin had an effect on early cyst survival over time.

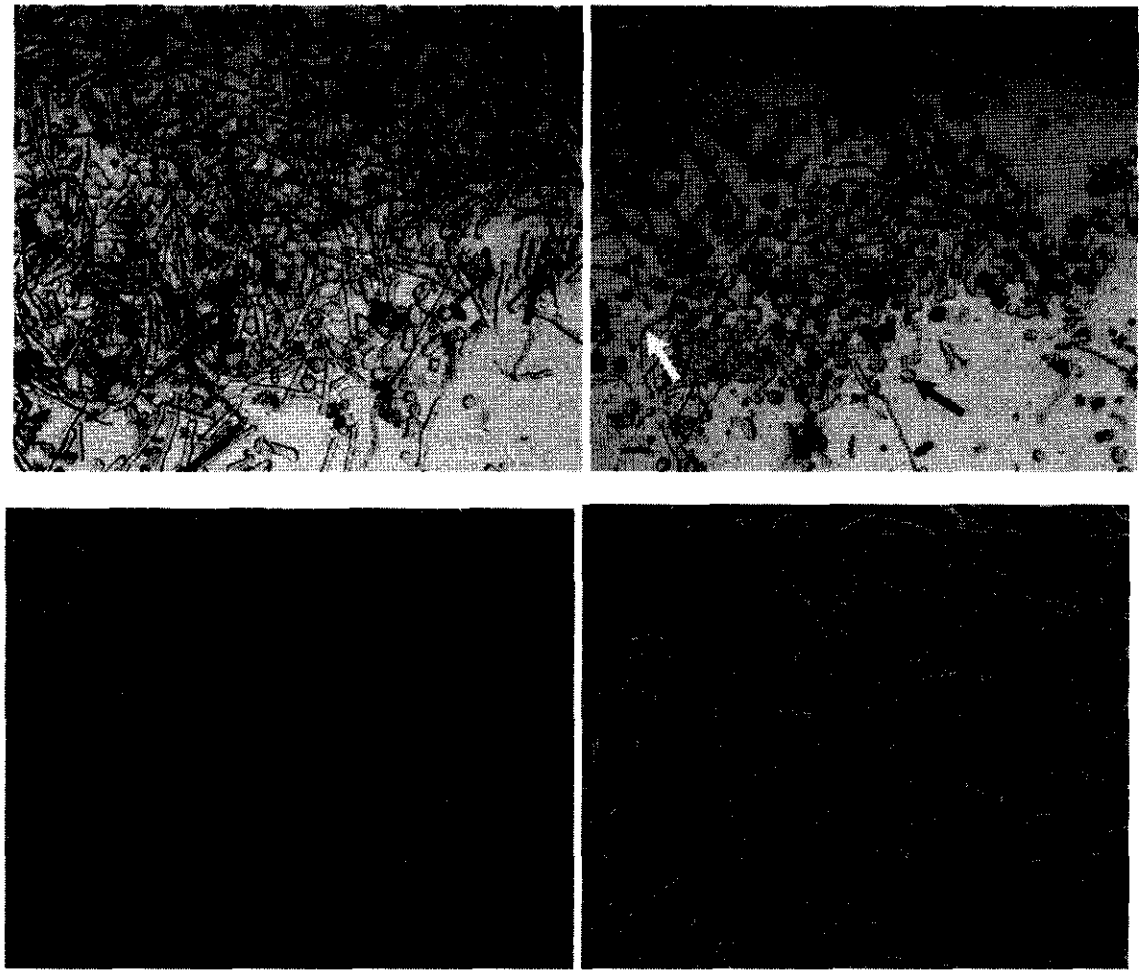

Figure 6. Isolated cysts over after 24 hours and 96 hours in culture supplemented with insulin. (a) Cysts after 24 hours in culture, note the appearance of a coiling cyst (black arrow). (b) Cysts after 96 hours in culture. Note more elongating cysts have a cystic bulge (white arrow). In addition coiling cysts were apparent (black arrows). (c) Phase contrast micrograph of cysts after 24 hours in culture. Note the appearance of degenerating cysts (black arrow) as well as early elongating cysts (white arrows). (d) Phase contrast micrograph of isolated cysts after 96 hours in culture. Note most early cysts have degenerated. Only post meiotic cysts are still visible (black arrows). Scale bars = 100 μm in all panels.

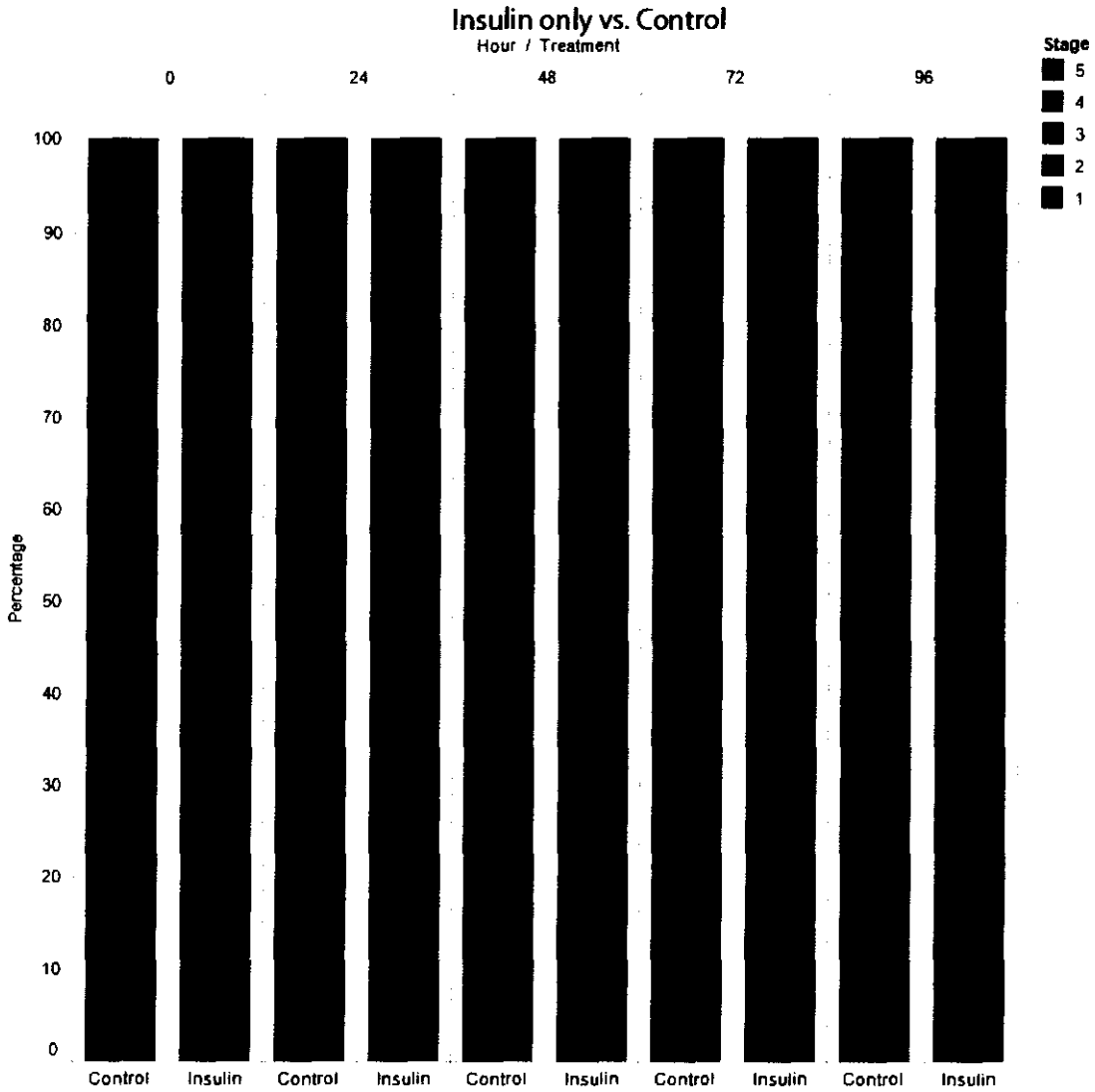

Figure 7. Percentage of viable cysts at different stages of development in cultures treated with insulin compared to non-treated over 96 hours. (1) Spermatogonia (in purple) (2) Primary spermatocytes (in dark blue) (3) Secondary spermatocytes/Round spermatids (in light blue) (4) Elongating and individualizing cysts (orange) (5) Coiling cysts (red). Data from a series of experiments were pooled.

iii. Insulin + Glutathione

Cultures treated with GSH + insulin showed similar qualitative results as those treated with each reagent independently. When initially isolated, cysts in advanced stages of differentiation were not observed. Cyst maturation was observed by the appearance of cystic bulge along elongated cysts and cysts at the coiling stage (Figs. 8a and 8b). Motile sperm was also apparent in GSH + insulin cultures.

After 24 hours the number of meiotic and post-meiotic cysts increased, whereas the number of mitotic cysts continued to decrease (Table 3 and Fig. 9). By day four, there was no significant difference in the percentage of mitotic cysts in treated vs. non-treated cultures (Fig. 9). In the normalized data, the ratio of early cysts in treated cultures to those in non-treated cultures was less than 1.0 after 24 hours. Even though the ratio of treated/non-treated was 1.3 after 48 hours, it decreased to 1.1 by day four (Fig. 10 bottom panel). Furthermore, in comparison to the non-treated cultures (Table 4), the number of meiotic cysts continued to decrease after 48 hours (Table 3). A greater number of cysts at the coiling stage were observed in the non-treated cultures compared to cultures treated with GSH + insulin after 48 hours (Tables 1 and 4). After 48 hours, the non-treated cultures had 10.5% of cysts at the coiling stage, while only 6.5% of cysts were at the coiling stage in the treated cultures (Fig. 11). Interestingly, this trend did not continue up to 96 hours. The percentage of cysts at the coiling stage in both treated and non-treated cultures were similar by day four (Fig. 9). These results suggest that cysts at all stages of development in treated cultures performed comparable to those in the non-treated cultures after 96 hours.

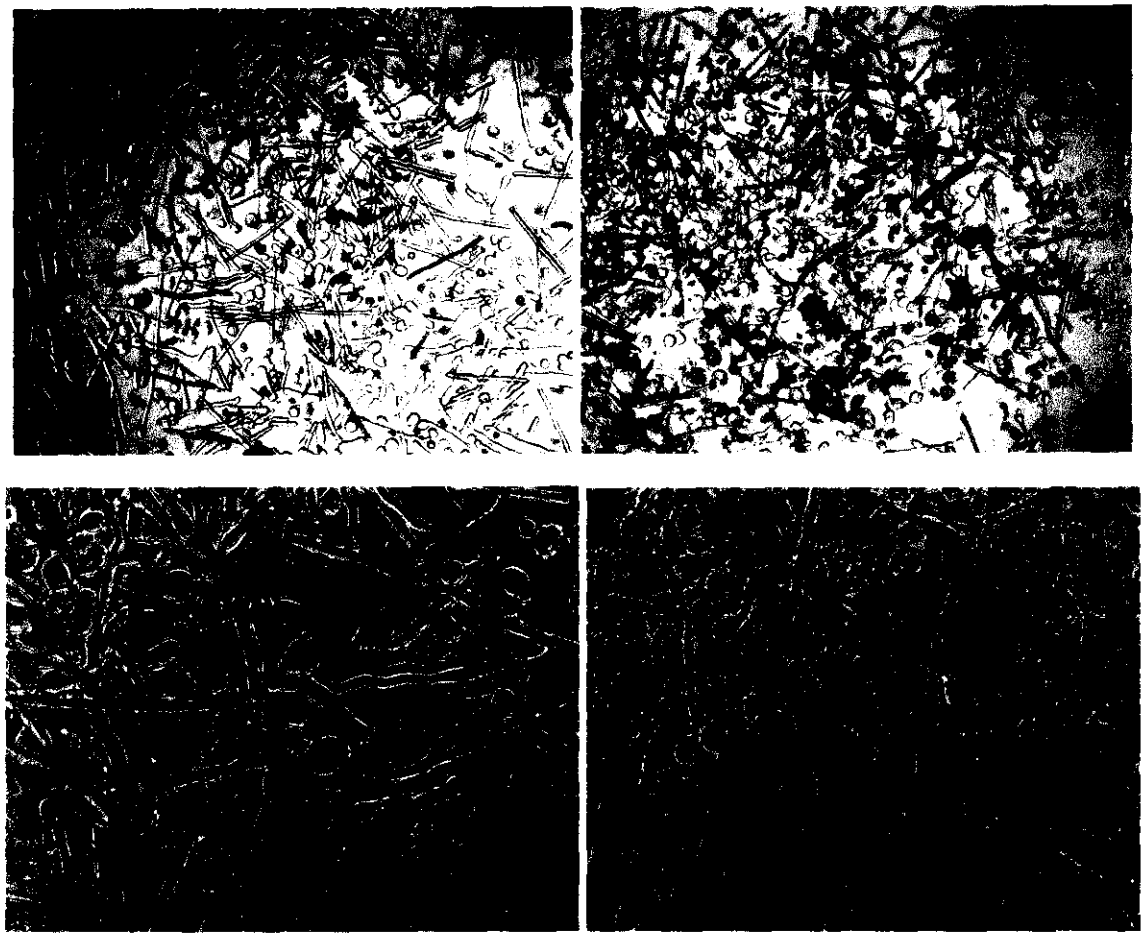

Figure 8. Isolated cysts after 24 hours and 96 hours in culture supplemented with GSH + insulin. (a) Cysts after 24 hours in culture, note the appearance of a coiling cyst (white arrow) and a cyst with a cystic bulge, indicative of individualization (black arrow). (b) Cysts after 96 hours in culture. Note increase in cell debris due to cyst degeneration. Coiling cysts were apparent (black arrows). (c) Phase contrast micrograph of cysts after 24 hours in culture. (d) Phase contrast micrograph of isolated cysts after 96 hours in culture. Note most early cysts have degenerated. Scale bars = 100 μm in all panels.

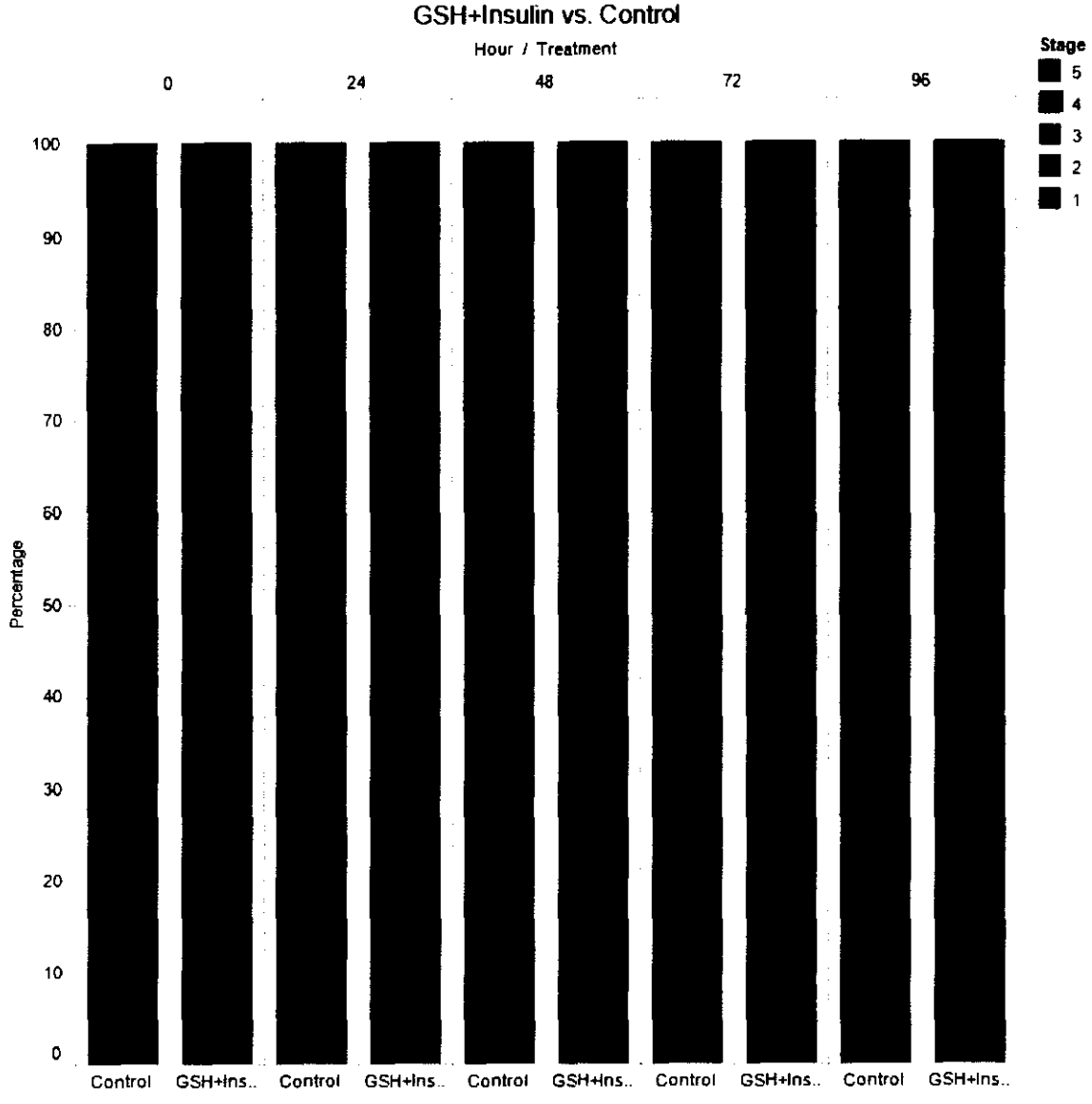

Figure 9. Percentage of viable cysts at different stages of development in cultures treated with GSH + insulin compared to non-treated over 96 hours. (1) Spermatogonia (in purple) (2) Primary spermatocytes (in dark blue) (3) Secondary spermatocytes/round spermatids (in light blue) (4) Elongating and individualizing cysts (orange) (5) Coiling cysts (red). Data from a series of experiments were pooled.

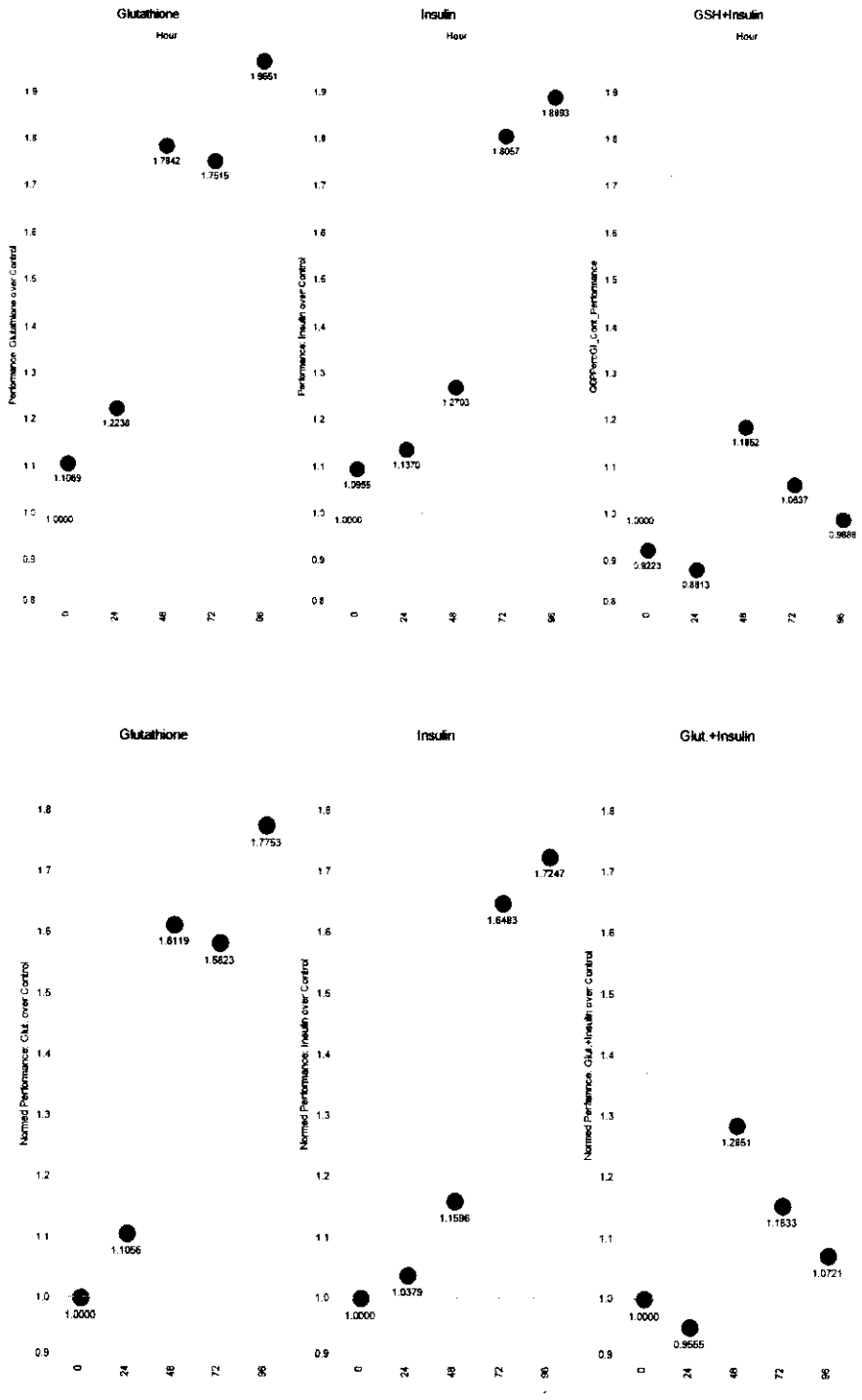

Figure 10. Performance of mitotic cysts in cultures over 96 hours treated with GSH only, insulin only, and GSH+insulin compared to non-treated cultures. Top panel raw data. Bottom panel normalized data.

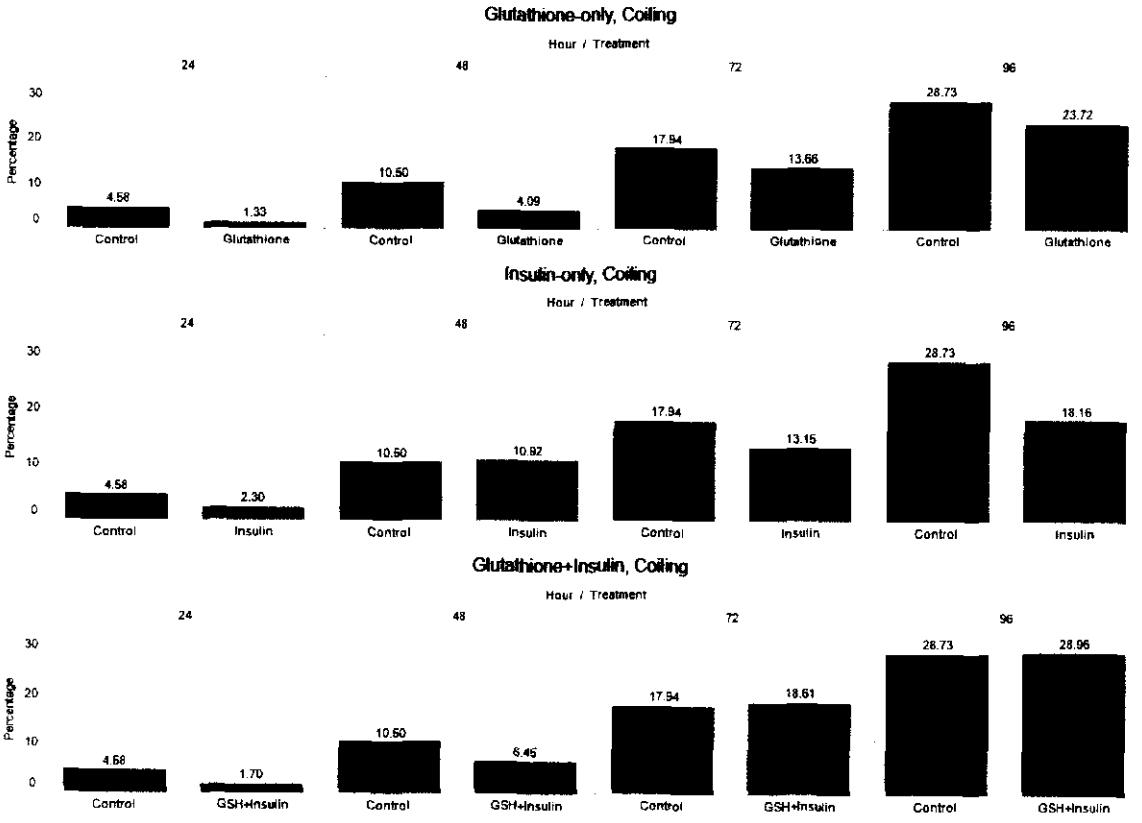

Figure 11. Coiling cyst performance between 24 hours and 96 hours in treated and non-treated cultures. GSH only (top), insulin only (middle), GSH + insulin (bottom).

iv. Quantification and statistical analysis of cyst growth

Cyst maturation was defined as development of the cyst up to the individualization stage which has the characteristic cystic bulge along the length of the cyst (Fig. 12b). The survival rates of the cysts were quantified based on how many viable cysts were present in culture per experiment each day; Spermatogonia (SG), primary spermatocytes (PS), secondary spermatocytes/round spermatids (RS), individualizing (IC) and coiling stage (CC) (Fig. 12). Spermatogonia that were undergoing mitotic divisions were identifiable based on the number of spermatogonia cells per cyst. Cyst cells at the 8-cell and 16-cell stage were counted as spermatogonia, but those at the 1-4 cell stage were not quantified in this study because they were difficult to distinguish morphologically. Once encapsulated within a cyst, the germ cell in *D. pseudoobscura* undergoes five synchronous mitotic divisions resulting in a cyst with 32 spermatogonial cells that are interconnected via cytoplasmic bridges (Sharer *et al.*, 2008). The cysts then undergo a growth phase, where the cell increases in size. The nuclei of the germ cells become smaller in size in post-mitotic cysts (Fig. 12a: RS). The results for each series of experiments for cultures with glutathione only, insulin only, insulin + glutathione and non-treated are summarized in Table 1-4.

A Student's t-test was performed comparing treated cultures to non-treated cultures (Table 5). In cultures treated with GSH, p values were statistically significant after 24 hours, with the exception of 72 and 96 hours. In cultures treated with insulin, p values were statistically significant only at 72 hours. Moreover cultures treated with GSH + insulin showed no statistical significance over four days, indicating that there was no

difference between non-treated cultures and cultures treated with GSH + insulin. Note that the cultures treated with GSH-only and insulin-only trend more closely towards a statistically significant difference from untreated cultures over insulin + GSH-treated cultures.

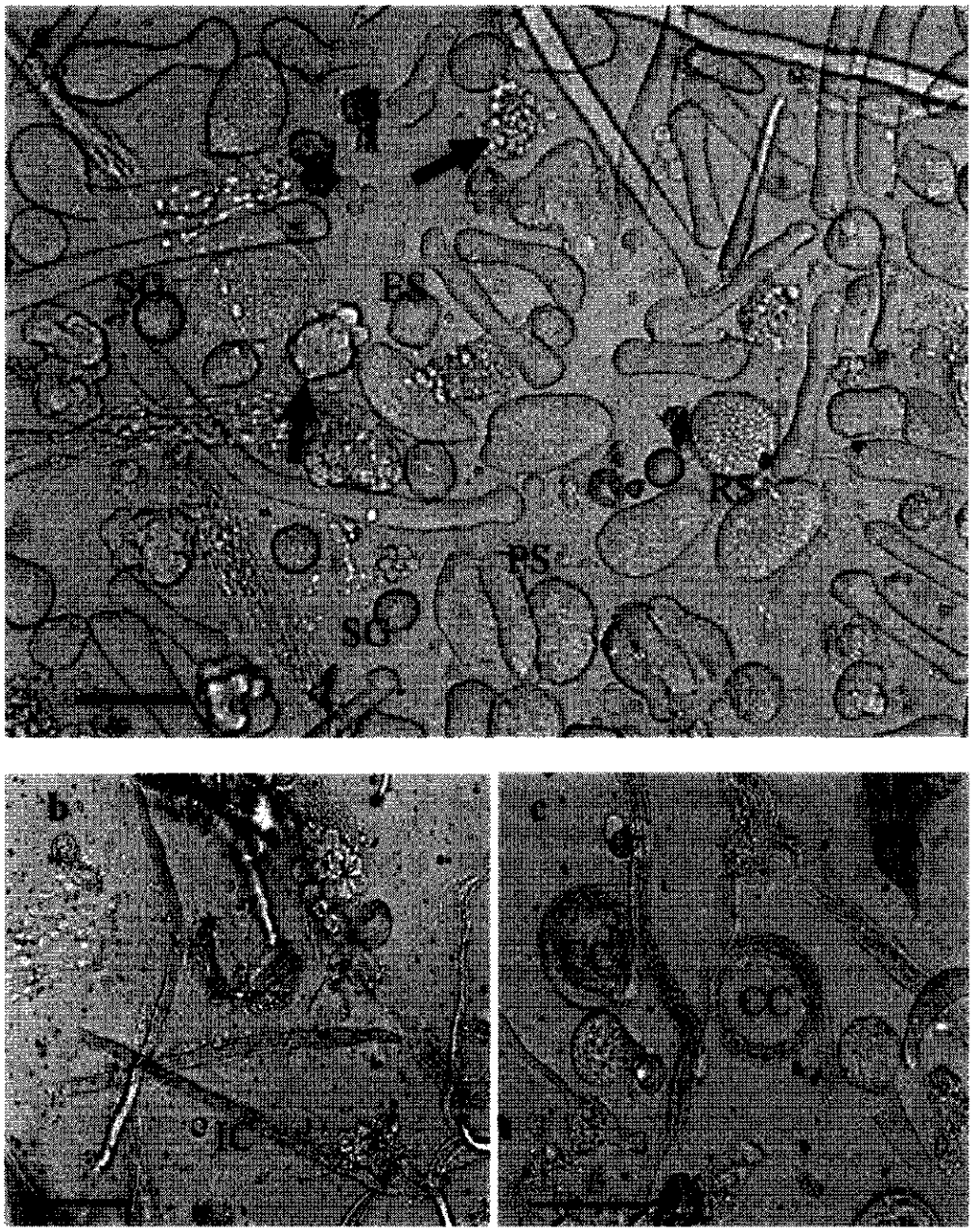

Figure 12. Spermatogenic cysts at 72 hours in culture treated with glutathione (a-c). a) Cysts at different stages of differentiation were observed. Degeneration of cysts was apparent (black arrows). SG: spermatogonia, PS: primary spermatocyte, RS: round spermatid, ES: early elongating cysts b) Cyst at the individualization stage of development. IC: individualizing cyst. C) Coiled cysts (CC). Scale bars = 100 μm in all panels.

Glutathione ONLY - POOLED DATA					
	Day 0	24 hours	48 hours	72 hours	96 hours
Spermatogonia	172	117	123	77	45
Primary spermatocytes	429	272	125	60	48
Secondary spermatocytes+Round spermatids	98	156	180	182	145
Elongating Cysts	581	721	581	528	344
Coiling Cysts	0	17	43	134	181
Total	**1280**	**1283**	**1052**	**981**	**763**

Table 1.Quantification of cyst development into advance stages *in vitro* treated with glutathione. There were no individualizing or coiled cysts at the initiation of the cultures.

Insulin ONLY - POOLED DATA					
	Day 0	24 hours	48 hours	72 hours	96 hours
Spermatogonia	172	137	70	56	35
Primary spermatocytes	328	132	73	36	25
Secondary spermatocytes+Round spermatids	73	93	153	58	54
Elongating Cysts	503	571	463	405	305
Coiling Cysts	0	22	93	84	93
Total	**1076**	**955**	**852**	**639**	**512**

Table 2.Quantification of cyst development into advance stages *in vitro* treated with insulin. There were no individualizing or coiled cysts at the initiation of the cultures.

Glutathione and Insulin - POOLED DATA					
	Day 0	24 hours	48 hours	72 hours	96 hours
Spermatogonia	202	96	75	32	21
Primary spermatocytes	244	135	78	35	15
Secondary spermatocytes+Round spermatids	133	165	131	74	53
Elongating Cysts	561	644	630	502	328
Coiling Cysts	0	18	63	147	170
Total	***1140***	***1058***	***977***	***790***	**587**

Table 3.Quantification of cyst development into advance stages *in vitro* treated with insulin + glutathione. There were no individualizing or coiled cysts at the initiation of the cultures.

Non-treated- POOLED DATA					
	Day 0	24 hours	48 hours	72 hours	96 hours
Spermatogonia	324	189	99	54	30
Primary spermatocytes	504	222	91	42	19
Secondary spermatocytes+Round spermatids	163	226	229	147	90
Elongating Cysts	961	946	868	745	424
Coiling Cysts	0	76	151	216	227
Total	**1952**	**1659**	**1438**	**1204**	**790**

Table 4.Quantification of cyst development into advance stages *in vitro* with no treatment. There were no individualizing or coiled cysts at the initiation of the cultures.

Percentage of Spermatogonia+Primary spermatocytes	0 hours	24 hours	48 hours	72 hours	96 hours
GSH-Experiment 1	46	32	21	12	8
GSH-Experiment 2	46	29	27	24	20
GSH-Experiment 3	50	30	25	9	10
GSH-AVG	47.33	30.33	24.33	15.00	12.67
GSH-SEM	1.33	0.88	1.76	4.58	2.73
Insulin-Experiment 1	40	38	21	18	20
Insulin-Experiment 2	47	26	14	14	7
Insulin-Experiment 3	50	23	11	12	10
Insulin-AVG	45.67	29.00	15.67	14.67	12.33
Insulin-SEM	2.96	4.58	2.96	1.76	3.93
Insulin+GSH-Experiment 1	42	31	23	5	4
Insulin+GSH-Experiment 2	35	26	18	10	7
Insulin+GSH-Experiment 3	40	20	13	10	7
Insulin+GSH-AVG	39.00	22.33	15.33	8.33	6.00
Insulin+GSH-SEM	2.31	3.33	2.73	1.67	1.00
p-values (treated compared to non-treated cultures)	0 hours	24 hours	48 hours	72 hours	96 hours
GSH only	0.160	0.013	0.000	0.116	0.038
Insulin only	0.460	0.312	0.339	0.025	0.104
GSH+Insulin	0.244	0.219	0.106	0.063	0.781

Table 5. Statistical analysis of treated cultures compared to non-treated cultures over 96 hours. A p value less than or equal to 0.05 indicates statistical significance.

II. Acridine orange and DAPI staining of cultured and adult cysts

The integrity of the chromatin in cultured sperm as compared to sperm obtained from adult cysts was analyzed using acridine orange (AO) staining. Qualitatively, no difference was detected in the green fluorescence obtained from both cultured cysts and cysts from adult males (Figs. 13b and c). Green fluorescence indicates binding of acridine orange to double-stranded DNA. Two sperm morphologies were observed in both cultured cysts and cysts obtained from adult males- a long sperm morph termed eusperm and a short sperm morph termed parasperm. The short sperm shown in Fig 13b averaged 84.6 +/- 3μm in total length, while their nuclei averaged 11.3 +/- 2 μm in length. The nuclei of the parasperm stained more intensely with acridine orange compared to the eusperm (Figs. 13b and c). This possibly indicates that the chromatin might be less compacted in the parasperm morphs which would allow the stain to have better access to the DNA for binding. The two sperm morphologies observed in both cultured cysts were confirmed using DAPI staining (Fig. 14). Both the parasperm and the eusperm were detected after 72 hours *in vitro*, as well as *in vivo*.

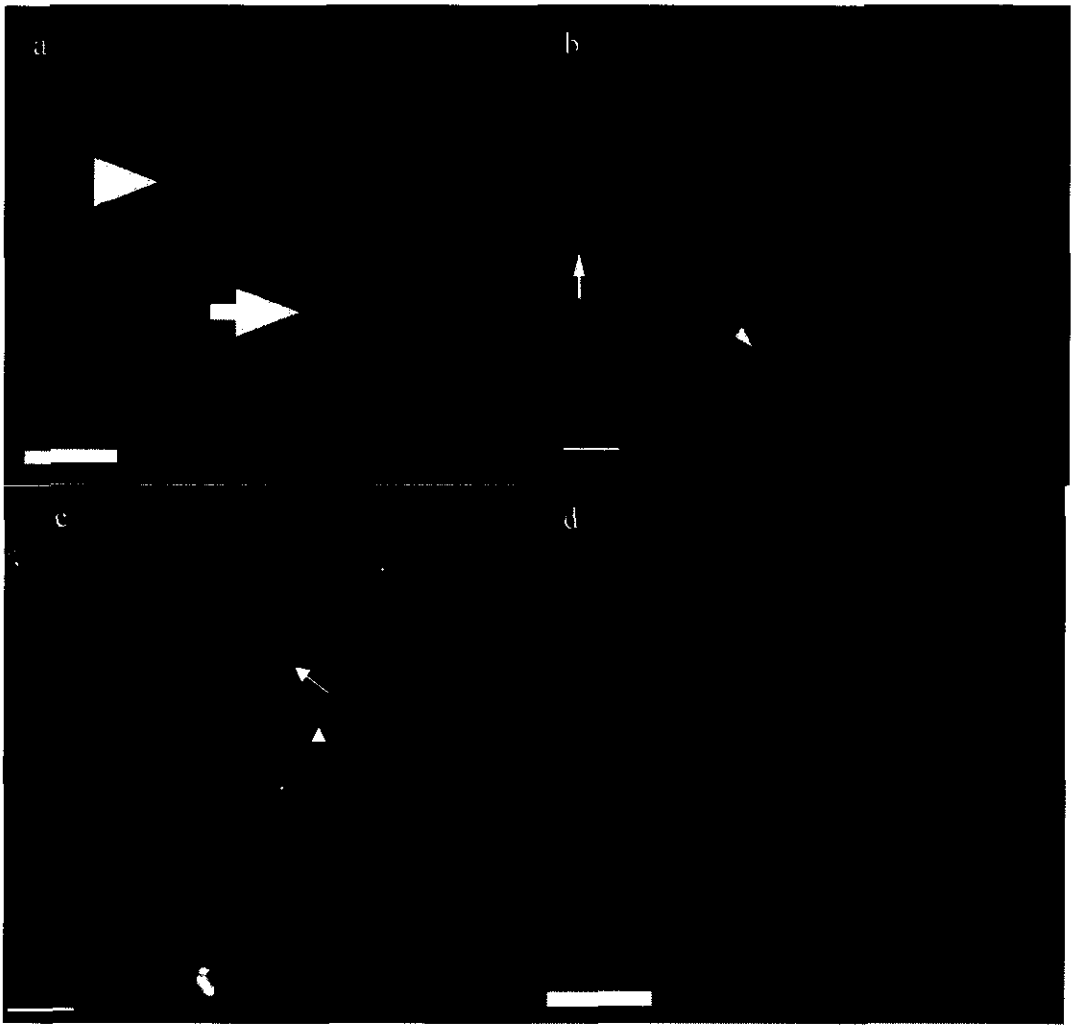

Figure 13. Acridine orange staining of cysts from three-day cultures and adult *D. pseudoobscura.* (a and b) Cysts and mature sperm after three days in culture. (a) Coiling cysts. The nuclei indicated by the arrowhead appear to be longer than the nuclei seen in the cyst indicated by the arrow. (b) Mature sperm that have burst from encapsulating cysts. Two sperm morphs are apparent; a eusperm is indicated by the white arrow, a parasperm is indicated by the arrowhead. (c and d) Mature sperm and cyst from *D. pseudoobscura* adult. (c) Mature sperm from adult testes. Two sperm morphs are apparent; a eusperm is indicated by the white arrow, a parasperm is indicated by the arrowhead. (d) Early elongating cyst. Nuclei have started to transform. Scale bar for panel a= 25 μm, b= 40 μm, c= 80 μm, d= 25 μm.

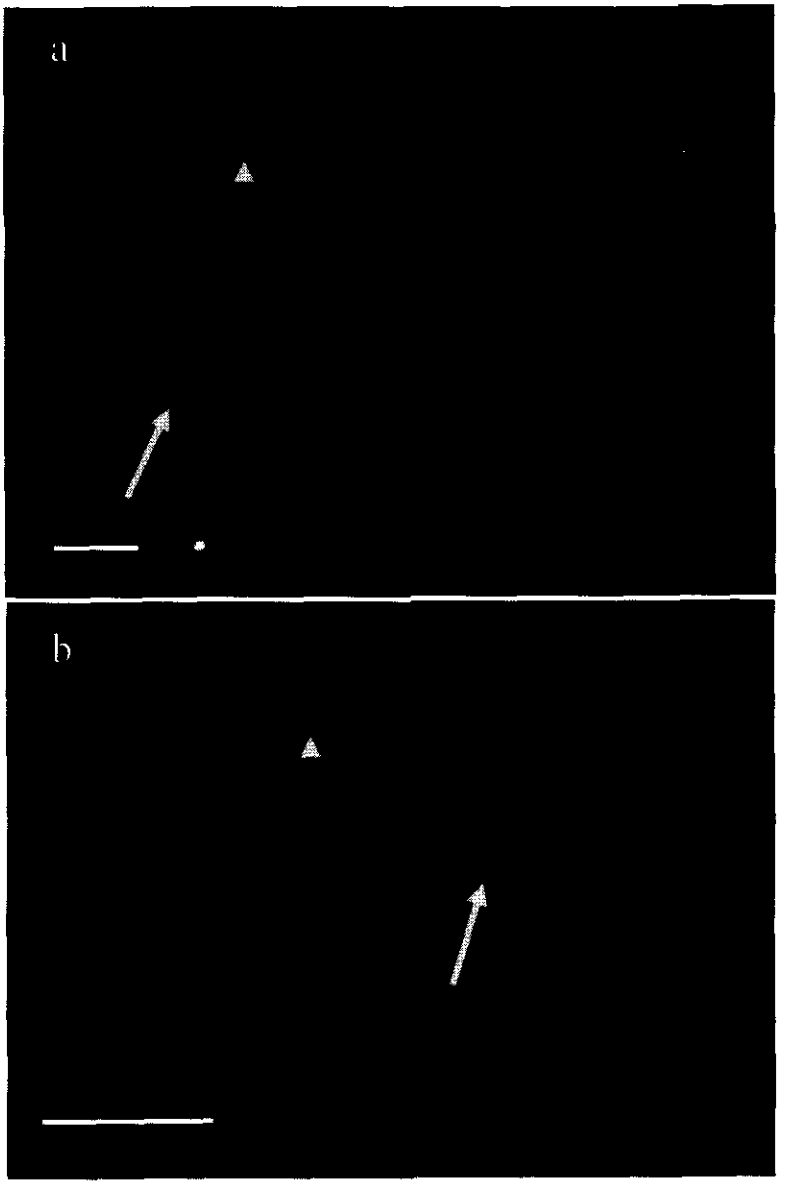

Figure 14. DAPI staining of individual sperm after 72 hours in culture. Eusperm indicated by the white arrows, and parasperm indicated by the arrowheads. Scale bars 100 μm in all panels.

DISCUSSION

i. Treatment of isolated cysts *in vitro*

Our results indicate that treatment of cysts with glutathione only, insulin only, and glutathione + insulin supported their growth to the elongated and motile form *in vitro*. Also, in non–treated cultures cyst maturation as well as motility was consistently achieved. However we sought to determine the effect of GSH and insulin treatments on the overall survival of spermatogenic cysts, and the survival of early cysts specifically. Cysts at the spermatogonia and primary spermatocyte state frequently degenerated in our previous study (Njogu *et al.* 2010). Quantitative analysis showed that the addition of glutathione only to the cultures had a greater effect on the survival of early spermatogenic cysts compared to cultures treated with insulin only, and glutathione + insulin, after 96 hours *in vitro*. Moreover, cultures treated with glutathione + insulin performed in a similar manner to control cultures that were not treated. In addition, our initial results on sperm chromatin integrity using acridine orange staining indicate that qualitatively there is no difference between chromatin integrity in cultured sperm and sperm obtained from adult testes.

D. melanogaster has been an animal model for developmental and genetic studies since the early 1900s, and spermatogenesis in this species of *Drosophila* has been described in great detail (Tokuyasu *et al.* 1972; Hardy *et al.*, 1979; Fuller *et al.* 1993). Our results affirm that *D. pseudoobscura* provides an excellent system for the investigation of spermatogenesis *in vitro* based on the high rates of success in cyst culture.

Moreover, the fact that its genome has been fully sequenced (Richards *et al.* 2005) makes it an ideal species for various studies. Our results for the culture of cysts isolated from *D. pseudoobscura* testes resemble germ cell development and differentiation in early reports of *in vitro* culture of *Drosophila* spermatogenic cysts with some important differences. Cross and Shellenbarger (1979) and Liebrich (1981) reported a low yield of fully elongated cysts, and motility was never observed. Fowler and Johannisson (1976) did not observe sperm motility in culture using *Drosophila hydei*. It was unclear why motility did not occur *in vitro*, however they hypothesized there may be a developmental factor or hormone in the *in vivo* system that functions to initiate spermiogenesis, which was absent in the *in vitro* culture. This hypothesis is contrary to our results in that motility was consistently observed in cultures in our previous and present work.

Recent studies have successfully investigated *in vitro* sperm culture in *D. melanogaster*. Noguchi and Miller (2003) used this system to analyze the role of actin in the individualization process in *D. melanogaster*. As previously mentioned, adult testes were used as starting material for Noguchi and Miller's cyst culture. The use of adult testes results in a high degree of cysts that undergo individualization and coiling. However, cysts at advanced stages of development at the onset of culture are not favorable for *in vitro* analysis of the molecular mechanisms involved in nuclear transformation, which occurs from the round spermatid stage to the elongation. Our studies utilized pupal testes because we are particularly interested in the transformation of the sperm nucleus.

Kawamoto *et al.* (2008) used larval testes from *D. melanogaster* as a starting material for the study of *Drosophila* spermatogenesis *in vitro*. They showed that sperm development and motility can be achieved in culture using testes from early and late larva. In our hands isolation of cysts from larval testes was unsuccessful because the testes are delicate at this stage, and thus easily damaged. Additionally, the testes are not pigmented in the larval stage and are therefore difficult to locate in the larval abdomen.

Glutathione is a water soluble tri-peptide that acts as a reducing agent to slow, prevent the oxidation of molecules (Sies 1999; Hwang *et al.* 1991). It consists of glutamate, cysteine, and glycine amino acid residues, and a thiol group, indicated by the red circle in the structure below, which participates in redox reactions. During oxidation, electrons are transferred to a molecule producing free radicals that start chain reactions that damage cells. Glutathione directly neutralizes free radicals and reactive oxygen compounds (Sies 1999; Hwang *et al.* 1991).

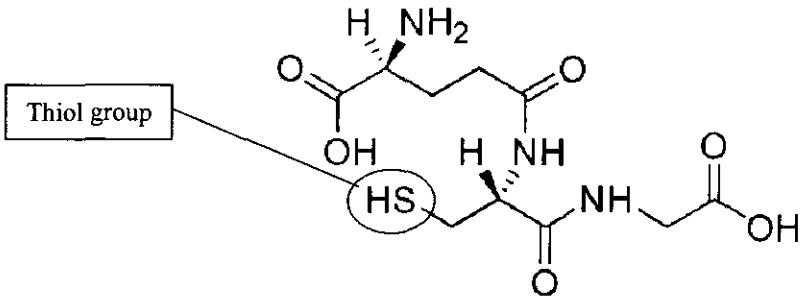

Figure 15. Reduced Glutathione. Adapted from Sigma Aldrich 2010

Our results showed that GSH had a significant effect on the survival of early cysts in culture between 24 and 48 hours, and continued up to 96 hours. We suggest that there was an improved survival of cysts at the 8, 16, 32 cell stage with the addition of the

antioxidant, which would result in a less frequent bursting of cysts observed in our previous work. In addition, we speculate that cysts at the 1-4 cell stage are also surviving in the treated cultures compared to the non-treated cultures, which would explain the higher number of cysts seen in our results. Presently we have no methodology to quantify cysts at the 1-4 cell stage.

Seven genes encoding insulin-like peptides (ILPs) have been identified in *D. melanogaster* genome (Gronke *et al.* 2010). These peptides are synthesized in clusters of medial neurosecretory cells in the *Drosophila* brain, but are expressed in various areas of the fruit fly. For example DILP7 is expressed in neurons that innervate the female reproductive tract (Gronke *et al.* 2010). An ILP receptor (insulin receptor, InR) and its downstream signaling cascade are well conserved in Drosophila. To understand the biological effects of insulin on cell growth and differentiation, Fernandez *et al.* (1995) genetically cloned *Drosophila* homologs of the insulin signaling pathway. They reported that loss of function mutations in the *inr* gene lead to embryonic lethality.

It has also been reported that altering the activity of the *Drosophila* insulin receptor changes the size and number of cells in the organism during development, which suggests insulin regulation of body size (Brogiolo *et al.* 2001). Ueishi *et al.* (2009) reported that GSC maintenance and spermatocyte growth were affected by insulin signaling in *Drosophila* mutants. Inhibition of insulin signaling resulted in a lower abundance of germline cells in *Drosophila* testes. In addition, spermatocytes growth was compromised in mutants that lacked insulin signaling, suggesting that insulin signaling plays a role in inducing the growth seen in primary spermatocytes (Ueishi *et al.* 2009).

Our results indicate that insulin had an effect on the survival of early cysts in culture between 48 and 72 hours, in comparison to GSH which had an effect between 24 and 48 hours. It appears that insulin supported the development of early cysts because quantitatively their degeneration was less frequent compared to those in the non-treated cultures. On the other hand cultures treated with insulin did not out-perform those treated with GSH. Possibly, the increase in performance of treated over non-treated cultures observed between 48 and 72 hours can be attributed to the addition of fresh culture media, which contained insulin, after 48 hours.

Interestingly, in cultures treated with GSH + insulin the ratio of early cysts compared to those in non-treated cultures was 1.1 after 96 hours. This indicates there was no synergistic effect over time when both agents were used. A possible explanation can be derived from the structure of GSH and insulin. As mentioned previously, GSH contains a thiol group that participates in the reduction of other molecules. The bovine insulin used in our study (Fig. 16) contains three disulphide linkages produced by cysteine amino acid residues.

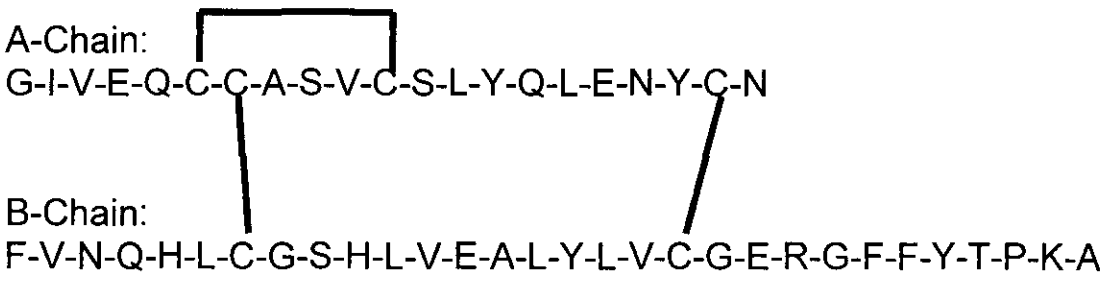

Figure 16. Bovine Insulin. Adapted from Derewenda *et al.* 1993 and US Pharmacopoeia 1995.

The disulphide bonds of certain proteins are often unreactive with thiols. However, certain proteins can be inactivated by reduction, in which the addition thiols initiate thiol-disulphide exchange reactions. Davidson and Hird (1967) investigated the reaction of glutathione with the disulphide bonds of purified proteins, including bovine serum albumin, pepsin, and insulin. These workers showed that the three disulphide bonds of insulin are reduced by glutathione. Proteolysis increased the reactivity of the proteins analyzed because the disulphide bonds within them became more accessible to glutathione. Native proteins under physiological conditions do not react with glutathione, which is found in every cell, because the disulphide bonds are structurally protected by steric hindrance or hydrophobic regions surrounding the bonds (Davidson and Hird, 1967). Our present study suggests that GSH and insulin interaction inactivated both agents as a result, so early cysts were not rescued from frequent degeneration observed in our previous work.

ii. Later cyst maturation *in vitro*

Cross and Shellenbarger (1979) reported that *in vitro* sperm coiling with *D. melanogaster* did not always follow the completion of individualization as *in vivo*. Additionally, the initiation of *in vitro* coiling occurred at variable stages of the individualization process. They also reported the *in vitro* development from meiosis to a coiled state was less than one-half the estimated *in vivo* time. Our results showed that in cultures treated with GSH 23.7% of cysts were at the coiled stage, while 28.7% of cysts were in the coiled stage in the non-treated (Fig. 11). Development of cysts at the coiled state in the non-treated cultures out-performed cultures treated with insulin only at 96

hours by a difference of 10.5%. This indicated that in the non-treated cultures, cysts progress to the coiling stage at a much faster rate. However in cultures treated with GSH + insulin, there was a 0.7% difference. This suggests that the addition of GSH and insulin independently retards the cyst maturation process *in vitro*, whereas the addition of GSH + insulin was similar to the events that occur in the non-treated cultures. The reason for the retardation of coiling in treated cultures is currently unknown.

iii. Mature sperm morphology and chromatin integrity

Species found in the obscura group within *Drosophila* are known to develop two sperm morphologies: sperm with short morphs are termed parasperm and long sperm are termed eusperm (Snook *et al.* 1994). Eusperm are capable of fertilizing *Drosophila* eggs, while the parasperm are not (Snook *et al.* 1998). We detected both sperm morphs in our *in vitro* cyst cultures. Initial analysis of total sperm length and sperm nuclei length indicate that cultured morphs are similar to adult morphs as reported in Snook *et al.* (1994). They reported a mean length of 362.5 +/- 1.5 μm for long sperm morphs with an average nuclei length of 56.6 +/- 0.39 μm. Short sperm morphs were reported as having a total mean length of 92.1 +/- 1.7μm with a mean nuclei length of 14.2 +/- 0.23 μm. Our results for *D. pseudoobscura* cultured sperm showed a parasperm mean length of 84.6 +/- 3 μm with a nuclei mean length of 11.3 +/- 2 μm. Moreover, the eusperm mean length was 327.5 +/- 6.5 μm with a nuclei mean length of 58.8 +/- 1.9 μm. Although our statistical analysis of the eusperm and parasperm is preliminary, our results are similar to results reported by Snook *et al.* 1994.

Our initial results on sperm chromatin integrity using acridine orange (AO) staining indicate no qualitative difference between chromatin integrity in cultured sperm and sperm in cysts obtained from adult testes. Acridine orange green fluorescence indicates binding of AO to double-stranded DNA, while red fluorescence (not shown in this study) indicates AO binding to single-stranded DNA (Evenson and Wixon 2006). However, the AO green fluorescence in parasperm was more intense than AO green fluorescence in eusperm. We suggest the increased intensity in AO fluorescence in parasperm could indicate that the stain has better access to the chromatin, possibly because the chromatin is less condensed. In addition, there is a possibility that the nuclei in the eusperm appeared less intensely stained because of the spatial arrangement of the DNA. We also suggest that a decrease in chromatin condensation may provide a mechanism for targeting parasperm for spermicide. Recent studies have suggested that the presence of parasperm in the female reproductive tract lessens the spermicidal effect the female tract has on the eusperm, thereby enhancing the survival of the eusperm (Holman and Snook 2008). DAPI staining confirmed the presence of the parasperm and eusperm in cultured *D. pseudoobscura* sperm as well as sperm from pupal testes. The fluorescence in the parasperm was also more intense that the fluorescence in the eusperm, as with acridine orange staining.

FUTURE STUDIES

D. pseudoobscura offers an easy and consistent *in vitro* culture system for studies where cellular transformation process can be manipulated. Future studies can utilize this culture system to investigate the mechanism of nuclear transformation. Since we are particularly interested in nuclear transformation, a possible future study could be a disruption of the microtubular perinuclear structure that surrounds the sperm heads known as the manchette. Additionally, we plan to perform dose response studies for both GSH and insulin in culture. The concentrations we chose to use in the current study were obtained from previous work done by Kawamoto *et al.* 2008 and Niki *et al.* 2006.

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