Germ cell death and their removal during initial stages of testicular ischemia and cryptorchidism: A comparative analysis

M M Misro, S P Chaki & Dinesh K Gautam

Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Mehrauli Road, Munirka, New Delhi 110067, India

Received 7 February 2005

Germ cell death and their removal from the seminiferous epithelium are common in the affected testis in conditions of unilateral ischemia or cryptorchidism; the similarities and differences, however, have not been studied between these two conditions. The present study was designed to examine the severity of the effect on testicular germ cells during the initial stages of both ischemia and cryptorchidism, which have significant implications on the restoration of fertility following surgical repair. Complete absence of spermatids was observed following 12 hr of ischemia as compared to 7 days of cryptorchidism. Germ cell removal in either case was in the direction of lumen to basement membrane leaving only a single layer of cells by 24 hr of unilateral ischemia as compared to 15 days of cryptorchidism. Levels of intratesticular testosterone was found lower in cryptorchidism (7 days) but not in ischemia till 24 hrs. Giant cells frequently observed in cryptorchid testis were absent in the ischemic seminiferous epithelium. There was a gradual increase in the number of apoptotic and non-viable cells; the latter was more than 95% by 24 hr of ischemia. In contrast, approximately 85% testicular cells were nonviable till 15 days of cryptorchidism. The ic peak representing the population of haploid cells was significantly reduced in cryptorchidism (7 days), while the peak was completely abolished by 24 hr of ischemia. Rise in the levels of oxidative stress in the affected testis was observed identically during the initial stages. These findings indicate that coupled with the rise in tissue oxidative stress, the number of apoptotic/nonviable germ cells was alarmingly high (>80%) by 15 days of cryptorchidism or 24 hr of ischemia. Restoration of complete spermatogenesis following surgical repair may not be possible in such cases because of these acute adverse effects.

Keywords: Cell death, Cryptorchidism, Germ cell, Ischemia, Testicular ischemia

Ischemia due to testicular torsion is regarded as a medical emergency in humans that may result in germ cell loss, aspermatogenesis and infertility. Compounding deleterious effects on spermatogenesis due to ischemia may not be entirely as a result of infarction because in cases where infarction has not occurred and the blood flow to the testis was immediately repaired to the pre-torsion levels, fertility was hardly improved either. Though Leydig and Sertoli cells functions did not shut down immediately after torsion, germ cells in the affected testis were found completely eliminated even after 30 days of torsion repair. It is therefore pertinent to know the stage beyond which the clinical repair hardly brings any improvement in spermatogenesis.

Similarly, cryptorchidism is one of the most commonly occurring congenital abnormalities affecting 1-5% of the boys with pathologies of testicular dysfunction. The effect of unilateral cryptorchidism on fertility has been more controversial as the undescended testis was shown to have adverse effects on the contralaterally descended testis. Even orchidopexy was reported to have little impact in improving the fertility probably because of the fact that the cryptorchid gonad was hardly contributing anything substantial to the overall sperm production. Thus, the irreversible damage in the affected testis during both ischemia and cryptorchidism must have occurred during the early stages, the analogies of which have not been studied in detail till date. In the present study, the process of germ cell death and removal have been examined in the seminiferous epithelium under early unilateral ischemia (till 24 hr) and cryptorchidism (till 15 days).

Materials and Methods

Animals, tissue preparation and histology—Adult male rats (Holzman strain), weighing 250-300 g, were maintained under controlled temperature (25 ± 2°C) and constant photoperiodic conditions (12:12 hr L:D) with food and water ad libitum. Animals under ether anesthesia underwent either unilateral ischemia or cryptorchidism. The contralateral testis in either case served as control.
The animals were killed by ether anesthesia following 1, 3, 6, 12 and 24 hr of ischemia or 1, 3, 5, 7 and 15 days of cryptorchidism. Twelve animals for each duration were used. Testes samples from 6 animals were immediately fixed in buffered formalin and dehydrated stepwise in graded ethanol. Blocks were made in paraffin and the sections (4 μm thick) were stained with hematoxylin and eosin or utilized for in situ end labeling (ISEL). Testes from the remaining 6 rats were decapsulated and divided into two parts. One part was utilized for in vitro cell isolation for determining cell viability, cell apoptosis and DNA flow cytometry, the other part was weighed, snap frozen and stored in liquid N2 till assayed for oxidative stress parameters.

Cell apoptosis—In situ Apoptosis Detection Kit (TA4625, R & D Systems, Inc, Minneapolis, USA) was utilized to identify apoptotic cells from other cells. Prefixed testicular cells or deparaffinized testis sections were utilized. To make the DNA accessible to the labeling enzyme, the cell membranes were permeabilized with proteinase K or Cytonin reagent. Cytonin was optimized to permeabilize isolated germ cell, and proteinase K was optimized for the permeabilization of dewaxed tissues prior to in situ detection of apoptosis. Endogenous peroxidase activity was quenched using H2O2. Next, biotinylated nucleotides were incorporated into the 3' ends of the DNA fragments by Terminal deoxynucleotidyl Transferase (TdT). The biotinylated nucleotides were detected by using streptavidin-horseradish peroxidase conjugate followed by the substrate, diaminobenzidine (DAB). The enzyme reaction generates an insoluble coloured precipitate where DNA fragmentation has occurred. DAB-stained samples were examined using a Nikon microscope image analyzer and photographed.

Cell viability—Germ cells were isolated from the control and treated testes following collagenase and trypsin treatment as described by Nagao10. Cells were washed in 10 mM PBS, stained with 0.4% trypan blue (Flow Laboratories, Irvine, Scotland) and examined under a phase contrast microscope. Stained cells represented the dead ones.

Flow cytometric analysis—Prevalence of haploid cells during different periods in the two conditions was examined through flow cytometry by the method of Spano and Evenson11. In brief, isolated cells were suspended in 10 mM PBS and fixed in 2 volume of cold methanol and mixed gently. After washes in PBS, RNase treatment was given at 37°C for 30 min and cells were stained with propidium iodide (50 μg/ml). Cells were examined for their DNA content in the Coulter EPICS® ELITE ESP flow cytometer (Coulter Corp., Miami, FL). WinMDI version 2.8 software was used for analysis.

Oxidative stress—A portion of the frozen testis was grinded under liquid nitrogen and a homogenate (20% w/v) was made in ice-cold PBS (50 mM, pH 7.4) containing 0.25 M sucrose. This crude homogenate (100 μl) was used for the lipid peroxidation assay. Following centrifugation, the supernatant (100 μl) was used for catalase assay. Rest of the supernatant was filtered through a 5 ml sephadex G-25 column and utilized for superoxide dismutase (SOD) or glutathione-s-transferase (GST) assay. The filtrate was diluted so as to obtain 25-50 μg protein/100 μl sample for SOD or 50-100 μg protein/100 μl for GST assay. Protein was estimated as described by Lowry et al12.

Lipid peroxidation—Lipid peroxidation in the crude homogenate (20%, w/v) was estimated by monitoring the formation of thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al13.

Enzyme assays—SOD activity was measured in the sephadex G-25 column eluted post mitochondrial supernatant (PMS) as described by Das et al14. Catalase activity15 was determined by measuring the rate of disappearance of hydrogen peroxide (60 mM) at 240 nm and glutathione-s-transferase activity was analyzed by the method of Habig et al16.

Hormone assays—Serum levels of LH and FSH were measured by RIA using reagents of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, USA). The minimal detection limit in the assay was 0.05 ng/ml for LH and 0.5 ng/ml for FSH. The intra and inter assay variations were 5 and 10%, respectively, in both the assays. RIA of testosterone was carried out following the methods of Abraham17 with modifications.

Statistical analysis—Data were analyzed by multiple two-tailed t-test and analysis of variance (ANOVA) using SPSS statistical software. Differences were considered significant at P<0.05.

Results

Adverse effects on seminiferous epithelium—Seminiferous epithelium depicting the adverse effects following 24 hr of ischemia and 15 days of cryptorchidism is shown in Fig 1. At this stage, a single layer of pyknotic germ cells could be seen in
both the conditions. However, initiation of adverse effects was evident by 3 hr of ischemia compared to 3 days of cryptorchidism (data not shown). Few seminiferous tubules in the cryptorchid testis even presented a spongy appearance with very few germ cells. Germ cell nuclei from the adluminal compartment in the ischemic testis were in a state of disintegration and at times it was difficult to identify them. Additional information on the state of germ cells in the seminiferous tubules came from observing isolated single tubule under a phase contrast microscope. While a tubule from control testis was seen with full sperms, a tubule from ischemic or cryptorchid testis was filled with free-floating cells and cell debris. There was a clear-cut detachment of cellular layers from the basement membrane within the seminiferous tubule by 24 hr of ischemia; fifteen days after cryptorchidism, the tubules appeared comparatively transparent with less number of germ cells (Fig. 2).

Germ cell apoptosis—Almost all germ cells close to the basement membrane were stained positive for
apoptosis following 24 hr of ischemia or 15 days of cryptorchidism (Fig. 3).

**Germ cell viability**—Viable germ cells declined gradually with increasing durations of ischemia or cryptorchidism. The decrease in cell viability was more acute in ischemia than in cryptorchidism. There was ~60% decrease in cell viability following 12 hr of ischemia which increased further to above 95% by 24 hr. By contrast, 15-18% cells were still found viable after 15 days of cryptorchidism (Fig. 4).

**Analysis of haploid cells by flow cytometry**—In the affected testis under both conditions, the number of haploid cells was very much curtailed. The data from flowcytometric analysis showed a significant reduction in the haploid peak after 7 days of cryptorchidism. The peak was found almost abolished by 24 hr of ischemia with the distribution shifting towards left, the area represented by cells, which were either dead or apoptotic (Fig. 5).

**Increase in oxidative stress**—Significant increase in oxidative stress in testicular tissue was evident as lipid peroxidation was found high either by 24 hr of ischemia or 7 days of cryptorchidism. Simultaneously, there was marked reduction in the activities of antioxidant enzymes such as superoxide dismutase, and catalase (Fig. 6).

**Intra-testicular testosterone**—Inratesticular testosterone, which declined significantly by 7 days of cryptorchidism, did not show any significant alterations till 24 hr of ischemia (Fig. 7). Gonadotropin levels remained unchanged in both the conditions during these early periods.

**Discussion**

Acute adverse effects like severe damages to the seminiferous epithelium, increase in the number of dead and apoptotic cells and significant germ cell loss with a virtually empty seminiferous tubule are some of the common features after a specific period of early ischemia or cryptorchidism. At different intervals in the present study, hormonal alterations were limited to low levels of intratesticular testosterone during cryptorchidism, but not in ischemia. Thus, failure in restoration of spermatogenesis in some of the affected testis after surgical intervention may be attributed to these changes in the initial periods, which sometimes prove to be completely irreversible.

![Fig. 2](image-url)

**Fig. 2**—Isolated seminiferous tubules filled with sperms in control testis (a, c) and with cell debris and round cells by 24 hr of ischemia (b) and 15 day of cryptorchidism (d). × 400.
Germ cells were severely affected during both early ischemia or cryptorchidism. Since ischemia induces hypoxic conditions, the effect on the germ cells was too severe and fast; it becomes therefore difficult to pinpoint the stage specific effect on spermatogenesis. By contrast, early cryptorchidism was seen to involve seminiferous tubules forming multinucleated giant cells with a large number of round spermatids in VI to VIII stages of the spermatogenic cycle. Cell removal through disintegration was in the direction of lumen.

Fig. 3—In situ end labeling (ISEL) of DNA in testicular sections. Control testis (a, during ischemia or c, during cryptorchidism) hardly displays any staining in the germ cells. Positive staining of germ cells close to the basement membrane was evident by 24 hr of Ischemia (b) and 15 days of cryptorchidism (d). ×400.

Fig. 4—A comparative analysis of viable germ cells (%) at various intervals during early periods of ischemia or cryptorchidism. Significant (P<0.05) reduction in number of viable cells was observed by 3 hr of ischemia and 3 days of cryptorchidism. P values: *<0.05, **<0.001
to the basement membrane in both conditions. Since hypoxia-induced germ cell apoptosis and necrosis extend rapidly to the spermatogonial cells, there is little chance of recovery if the period of ischemia extends by more than 3 hr at the maximum. An analogous period would be 3 days for cryptorchidism, though the process of spread of germ cell apoptosis was relatively slow in cryptorchidism. Germ cell apoptosis continues following surgical repair too as the first evidence of germ cell apoptosis was detected 4 hr after repair of testicular torsion of 1 hr duration.18

Hypoxia induces a fast rise in the number of dead and apoptotic cells. The present results show that by 24 hr of ischemia, the number of dead testicular cells was more than 95%; in contrast, however, 10-15% of the cells were found to be alive in the testis until 15 days of cryptorchidism. Considering the fact that there was only a single layer of cells lying close to the basement membrane, it is more likely that all these cells may not be viable by 24 hr of ischemia but may stain positive for apoptosis by ISEL technique. The ISEL technique showing labeling of necrosing cells has also been reported in some conditions.

It is interesting to note that early ischemia or cryptorchidism affects mostly the haploid cells first,

Fig. 5—Flowcytometry of testicular cells isolated in vitro, control versus ischemia (24 hr) and cryptorchidism (7 days). Four different cell populations corresponding to four different peaks, such as haploid cells like sperms as 1c, spermatids as 1c, diploid cells like spermatogonia, spermatocytes as 2c, and tetraploid cells like post-leptotene primary spermatocytes and G2M spermatogonia as 4c are identified in control (a). 7 days of cryptorchidism (b) reduced the 1c peak, and it was found abolished by 24 hr of ischemia (c).

Fig. 6—Evaluation of oxidative stress in ischemic and cryptorchid testes. There was significant rise in lipid peroxidation as shown in TBARS formation by 24 hr of ischemia and 7 days of cryptorchidism. Simultaneous decrease in the activities of superoxide dismutase and catalase was also observed. * P<0.001

MISRO et al.: GERM CELL DEATH IN TESTICULAR ISCHEMIA ETC.
Hormonal alterations have been attributed as yet. Intratesticular testosterone is characteristically high during normal spermatogenesis although no specific role for the same has been attributed as yet. Hormonal alterations have been earlier reported to be the prime cause for increased germ cell apoptosis in the seminiferous epithelium. There were no significant alterations in the serum gonadotropins and testosterone concentrations by 24 hr of unilateral ischemia or by 15 days of unilateral cryptorchidism (data not shown). Testosterone has been reported to prevent apoptosis in testicular germ cells. In the present study, the unchanged levels of intratesticular testosterone seems to have little impact in preventing germ cell apoptosis in ischemia, though a decline in its concentration may be a factor, stimulating germ cell apoptosis in cryptorchidism. The molecular pathway of apoptosis in the germ cells as a result of ischemia or cryptorchidism remains unclear and needs further study.

Elevated oxidative stress in the affected testis was the hallmark of both ischemia and cryptorchidism. In the present study, a significantly increase in oxidative stress was observed by 24 hr of ischemia and after 7 days of cryptorchidism. Increased lipid peroxidation further leads to a rise in the production of toxic ROS that adds to the vicious cycle of more peroxidation and damage to the existing testicular cells. ROS can induce apoptosis in germ cells. Since the levels of indigenous antioxidant enzymes like SOD and catalase were also on decline, supplementation with exogenous antioxidants may help to improve the overall condition in the seminiferous epithelium with or without surgical repair of the affected testis. However, this needs to be proved in subsequent studies.

The situation is not analogous if intratesticular testosterone levels are considered in both the conditions. Intratesticular testosterone is characteristically high during normal spermatogenesis although no specific role for the same has been attributed as yet. Hormonal alterations have been

![Graph showing decrease in intratesticular levels of testosterone during early ischemia (24 hr) or cryptorchidism (7 days).](image)

Fig. 7—Decrease in intratesticular levels of testosterone during early ischemia (24 hr) or cryptorchidism (7 days). *P<0.001

References


