Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 10 (2)

Stress and Testicular Integrity; Impacts of Cryptorchidism and Dexamethasone in Adult Wistar Rats (*Rattus Novergicus*)

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ABSTRACT
Impacts of cryptorchidism and dexamethasone (Corticosterone analogue drug) as forms of stressors on the testicular integrity of adult wistar rats were investigated in this study. Several forms of stressors had been implicated in male infertility as the hallmark consequence of hormonal dysfunction, primary spermatogenic failure, ejaculatory disorders, and obstruction and testicular dysfunction. Adult wistar rats were exposed to unilateral and bilateral cryptorchidism respectively; Two groups of Five (5) animals of the exposed, were treated with 10 mg/kg body weight of dexamethasone. The administration of dexamethasone was done for a period of seven (7) days following unilateral and bilateral cryptorchidism exposure. The animals were euthanized by cervical dislocation and testes exposed following midline abdominal incision. The right caudal epididymis was ligated for sperm analysis and the left testes fixed in Bouin's fluid for histological analysis. Significant reduction in the sperm motility, sperm count and abnormal sperm morphology characterized animals exposed to both unilateral and bilateral cryptorchidism. Spermatogonia degeneration, distortion in the spermatogonia lineage differentiation and abnormal widening interstitial spaces form the typical features of the cryptorchids testes group and cryptorchids testes group exposed to dexamethasone administration. However, control animals maintained the testicular integrity and the characteristic sperm analysis. Therefore, cryptorchidism and dexamethasone exposure contribute to the form of stressors that destruct spermatogonia population linage with consequent alteration in sperm characteristics causing increased in defective spermatozoa, promoting infertility among male.

Key Words: Male infertility, Cryptorchidism, Dexamethasone, Testes, Stressors and wistar rats

INTRODUCTION
Male infertility has been attributed to a variety of causes such as hormonal dysfunction, spermatogenic failure, ejaculatory disorders, obstruction and testicular dysfunction and scrotal temperature [1,2]. Cryptorchidism as part of testicular dysgenesis syndrome is related to genetic (trisomy), hormonal (defect in androgen action), and anatomical factors, with common defect in spermatogenic processes as consequence [3,4]. Several studies had linked cryptorchidism with reduced fertility and testicular cancer due to increased intra-abdominal pressure [5,6,7,8]. High intra -abdominal temperature in cryptorchid testis generates reactive oxygen species (ROS), causing oxidative stress and disrupt spermatogenesis implicated in male infertility [9]. Inability to achieve conception after one to two years of regular, unprotected intercourse defines infertility and about 15% of couples failed to achieve pregnancy within one year of unprotected sexual intercourse with the male factor account for about 50% in every 20% of couples [10,11].

Glucocorticoids (GCs) as being linked with increased oxidative stress in the cell and are class of corticosteroids, steroid hormones that exact their actions on cells by binding to the glucocorticoid receptor (GR) thereby formed an activated GR complex that up-regulates the expression of anti-inflammatory proteins and represses the expression of pro-inflammatory proteins[12,13]. Dexamethasone is a synthetic glucocorticoid whose potency to suppress the immune system is 20-30 times greater than that of hydrocortisone and binds strongly to the glucocorticoid receptor than cortisol and had been implicated in overproduction of reactive oxygen species [13,14]. This study is therefore designed to investigate the cytoarchitecture of the testis following exposure to two (2) major form of stressors; cryptorchidism and dexamethasone treatment in adult wistar rats.

MATERIALS AND METHODS
Experimental Animal: Twenty-Five (25) adult male wistar rats with an average weight of 130g±20g were obtained from the Faculty of Basic Medical Sciences Animal Holding, Osun State University, Osogbo. These animals were kept in the animal house of the Faculty of Basic Medical Sciences, Osun State University. The rats were on a daily basis fed with rat feed bought from Breed feeds and flour mill limited, Olaiye, Osogbo.

They had access to water *ad libitum*. Proper aeration was maintained by use of well-spaced and gauzed cages and a hygienic environment was ensured. The animal rooms were well ventilated with a temperature range of 25-27 under day/night 12/12 hours photoperiodicity.

Experimental Procedure: All protocols and treatment procedures were done according to the Institutional Animal Care and Use Committee (IACUC) guidelines and in accordance to the Faculty of Basic Medical Sciences Ethics Review Committee, Osun State University, Osogbo, Nigeria. The animals were selected randomly into Five (5) groups of Five (5) animals each (n=5), Group 1 (Control) received normal saline, Group 2 Unilateral Cryptorchidism (CRX) animals were exposed to surgical procedure for the suspension of the left testis in the lower abdominal region under controlled hygienic anesthesia, Group 3 Bilateral Cryptorchidism (CRX), animals were exposed to surgical procedure for the suspension of the both testes in the lower abdominal region under controlled hygienic anesthesia, Group 4 received 10 mg/kg body weight of Dexamethasone and was exposed to unilateral cryptorchidism (DEX+CRX), Group 5 received 10 mg/kg body weight of Dexamethasone and was exposed to bilateral cryptorchidism (DEX+Bi-CRX).

Unilateral and Bilateral Cryptorchidism: Adult male wistar rats were used for this experiment. The rats were anesthetized with chloroform, and a small incision was made in the right side of the lower abdominal region. Following the abdominal incision, testis was drag into the lower abdominal region and anchored to the inner lateral abdominal wall by a suture passing through the inguinal canal. Particular care was taken to avoid any contact with the testis during the procedure. In bilateral cryptorchidism, both testes were suspended in the lateral abdominal wall.

Drug Administration: Administration was done oro-gastrically by the use of oral cannula, 10 mg/kg body weight of dexamethasone was administered obtained from Ladoke Akintola University of Technology Teaching Hospital Pharmacy Department, Osogbo. The solution was freshly prepared each morning of administration and kept at 4^{o} C before use. The administration was carried out each day at about 8.00 am for the period of Seven (7) days following exposure to cryptorchidism.

Animals Sacrifice: All animals were euthanized by cervical dislocation twenty-four (24) hours after the last administration of dexamethasone, the caudal epididymis of the animal was excised following abdominal incision for semen analysis while the testis was excised and fixed in Bouin's fluid for histological examinations.

Semen Analysis: To determine the percentage of motile sperm, a drop of the sperm suspension was drawn using a rubber Pasteur pipette and placed on a Neubauer chamber, covered by a 22×22 mm cover slip and the percentage of motile sperm was evaluated. The concentration and total count of spermatozoa was estimated using a Neubauer hemocytometer. A fixed volume of the sample was withdrawn with a micro-pipette and delivered onto the edges of Neubauer chamber of hemocytometer. Both chambers of hemocytometer were scored and the average count was calculated. The count was done under a light microscope at ×400 magnifications and expressed as $\times 10^{\wedge 6}$/ml**.** Moreover, to examine the sperm morphology, a drop of the sperm suspension was placed on a glass slide and a smear was prepared. The smear was fixed in ethanol for 1 h, stained with hematoxylin and eosin, washed, dried, and examined with a light microscope at a magnification of ×100. Spermatozoa were counted and the percentage of abnormal sperm determined. Morphologic abnormalities of spermatozoa were categorized as: Head defect, including large, small, amorphous, vacuolated, double heads or any combination of these, Neck defects, including distended/irregular/bent midpiece, abnormally thin mid-piece or any combination of these and Tail defects, including absent tail, short, multiple, hairpin, irregular width, or coiled tails, tails with terminal droplets, or any combination of these. These finding were expressed as percentage (%) of morphological abnormal sperm.

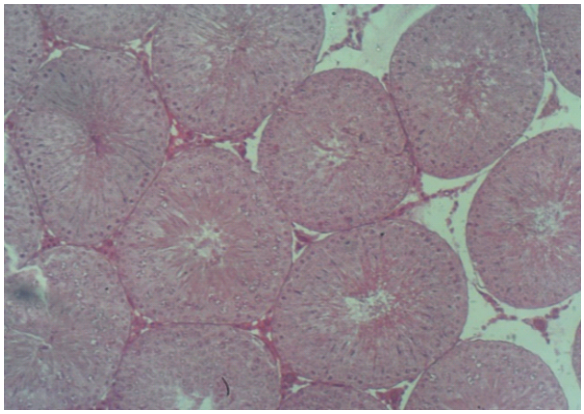
Statistical Analysis: All quantitative data were analyzed using GraphPad Prism® (version 6) soft-wares using ANOVA and Tukey's multiple comparisons test. Significance was set at $p<0.05$*. The results were represented in mean and standard error of mean (Mean ± SEM) respectively.

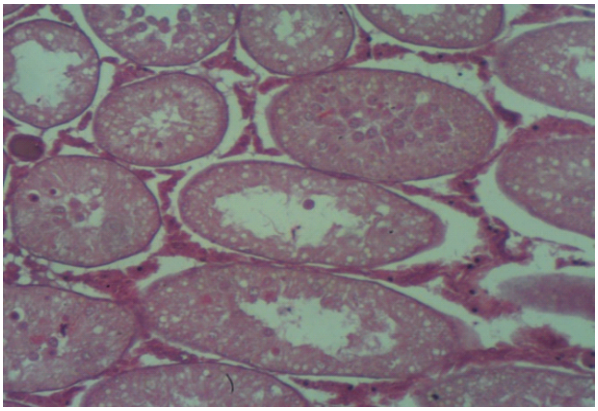
RESULTS

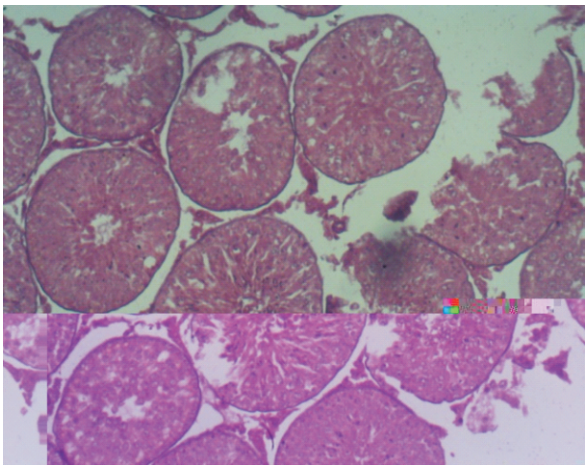
TOTAL SPERM COUNT (x10 ^{6}/ml)		SPERM MOTILITY (%)		SPERM MORPHOLOGY (%)	
GROUPS	MEAN±SEM	MOTILE	NON-MOTILE	NORMAL	ABNORMAL
CONTROL	121.04 ± 1.57	90.00 ± 2.03	10.00± 2.21	89.50 ± 1.24	10.05 ± 1.24
CRX	39.80 ± 1.31*	62.50 ± 0.53*	37.50 ± 0.62*	66.25 ± 2.10*	33.75 ± 1.38*
Bi-CRX	35.00 ± 3.46*	63.45 ± 1.74*	36.55 ± 1.49*	56.07 ± 2.71*	43.93 ± 2.35*
DEX+CRX	56.00 ± 1.14*	58.80 ± 0.96*	41.20 ± 0.92*	64.50 ± 1.92*	35.50 ± 1.77*
DEX+Bi-CRX	51.60 ± 2.52*	52.50 ± 0.93*	47.50 ± 1.08*	51.50 ± 2.04*	48.50 ± 2.61*

Data were presented as mean and standard error of mean (Mean ± SEM)
*($P<0.05$) – Statistically significant difference when compared across groups.

Histological Observations







Semen analysis: The sperm count, sperm motility and morphology were analyzed after the caudal epididymis of the rats was excised. Table 1 expressed the sperm characteristics observed in control animals, cryptorchids exposed animals and cryptorchid animals treated with the administration of dexamethasone. Sperm concentrations was significantly higher in the control animals, percentage normal motility and morphology were significantly highest compared with the cryptorchid animals treated with dexamethasone. However, sperm characteristics significantly altered in cryptorchid animals compared with the dexamethasone treated, significant reduction in sperm count and deformity in the basic structure of spermatocytes were observed.

Histological examination revealed rounded or oval seminiferous tubules with regular contour, the spermatogonia, Sertoli cells and the spermatid as shown in figure 1(A) in control animals. Testicular integrity preserved, the spermatogonia population well expressed in all stages, the germinal epithelia well demonstrated with the intact basement membranes and the interstitial spaces occupied by the leydig cells. The seminiferous tubules showed lightly stained Sertoli cells and the synchronous development and diffferentiation in the spermatogonia from the germinal epithelium to the adluminal compartment figure 2(A).

Proliferation of Sertoli cells and marked degeneration of the spermatogia population with the consequent reduction of the adlunimal compartment spermatocytes characterized the testes suspended in the lower abdominal region both in unilateral and bilateral cryptorchidism. Increased vacuolation along the line of proliferation and differentiation of spermatogia lineage and widening of the adlunimal compartment resulting from the loss of spermatogia in animals' testes exposed to heat form of stress "cryptorchidism" as seen in figure 1(B) and 4(B). Figure 2 (B) and (1C) showed as well in both unilateral and bilateral cryptorchidism accumulation of the connective tissue in the basement leading to the thickening in basement membrane; a characteristics of an age testes in rats.

Seminiferous tubules in cryptorchids showed markedly reduced number of differentiating cells with vacuolated cytoplasm and dark nuclei and testicular parenchyma appeared parked with tubules with extensive widening of lumen and interstitial space figure (1C) and (2C). More importantly in dexamethasone treated animals the basal membrane of the tubules was seen surrounded

by spindle shaped cells, interstitial space in-between the tubules contain Leydig cells (LC) having vesicular nucleus with prominent nucleolus, and abnormal widening of lumen and numerous Sertoli cells cause the degeneration of spermatogonia. Atrophy of the seminiferous tubules that consequently results in the widening of the interstitial spaces and the reduction in the population of the leydig cell as observed in the dexamethasone treated animals that were exposed to the cryptorchidism form of stress. Figure 2(C) presented the testicular architectures in dexamethasone treated animals which were exposed to cryptorchid form of stress, these demonstrated expression of large number of Sertoli cells, thickening of the basement membrane and widening of the interstitial spaces as well. Therefore, reduced spermatogenesis, shrinkage in the seminiferous tubules and widening interstitial spaces with reduced leydig cells characterized the histological features of the testes exposed to form of cryptorchids stress. Dexamethasone in particular enlarge the spaces between the seminiferous tubules and reduce the leydig population as observed in figure 2(D).

DISCUSSION

Cryptorchidism, hypospadias, testicular cancer and stressors have been associated with the decreased semen quality and quantity [15]. This study examined significant reduction in the sperm counts, motility and morphology in the sperm characteristics of animals exposed to cryptorchidism and dexamethasone. This is also in line with what occur in human; sperm density decreased in 30% of men with unilateral cryptorchidism, according to Lee and Huston, while in bilateral undescended testis 50% of patients showed decreased sperm densities [16]. Increased temperature in the lower abdominal region could have imposed negatively on the production and the survival of the spermatocytes in the adluminal compartment. This was evidence in the reduction in the spermatogonia population observed in the histological slide. Production and maturation of spermatocytes from the germinal epithelium to the adluminal compartment required lower temperature to the body temperature, which necessitate the contraction and relaxation of the dartos muscle of the scrotum in regulating the scrotal temperature to optimized the environment for the production and maturation of the spermatocytes.

The present study demonstrated experimental cryptorchidism induced germ cell apoptosis and generation of spermatogonia population at all stages of development and differentiation, as seen on bilateral and unilateral cryptorchidism which ultimately altered the semen quantity and quality compared with the control animals. Ng et. al., [19] noted atrophy of the seminiferous tubules in aged rats and thickening of the basement membrane, this research work as well noted vividly in the teste stained with Masson trichrome in animals exposed to stress both in hormonal stressor and mechanical stressor as seen in animals treated with dexamethasone and the animals exposed to cryptorchid.

Kubota *et al.,* [18] found the number of the leydig cells reduced in cryptorchidism and delayed appearance of primary spermatocytes with generally reduced total number of germ cells. Also noted is the failure of optimal maturation of germ cells associated is the tubular and interstitial damage detected in early months of life in patients with cryptorchidism [19].

This result is suggestive of the fact that induces oxidative stress which leads to spermatogenic arrest and modulation of the hypogonadal-pituatry-gonadal axis. Reports from previous studies agrees with the findings from this research [20,21]. The present results, consistent with earlier observations, indicate that dexamethasone also induces decreases in semen quality [10,22]. Pituitary gonadotropins and testis androgens are essential for the growth and differentiation of somatic cells in the testis as well as for the initiation and maintenance of spermatogenesis, including optimal germ cell growth and differentiation. In another study, [22] studied the effect of dexamethasone on Bax protein expression as an apoptotic protein in germ cells of 35 female mice and found that glucocorticoid compounds such as dexamethasone can come up with apoptosis and disruption in oogenesis process affecting pro-apoptosis proteins such as Bax.

Morphological studies have indicated furthermore, that in unilaterally and bilaterally cryptorchid rats, the abdominal testes showed marked apoptotic DNA cleavage as seen in the feugen DNA analysis when compared to the control group testes. Thus, cryptorchidism provides a valuable model for future elucidation of intra-testicular mechanisms triggering apoptosis. Continuous degeneration of spermatogonia with morphological features of the characteristic apoptosis occurs in adult rat cryptorchid testis and dexamethasone treated testis. In addition, experimentally induced unilateral cryptorchidism provides a useful model for study of the role of local factors regulating testis cell apoptosis [19].

Histological analysis from this study shows the seminiferous tubules appeared rounded or oval with regular contour, the spermatogonia were degenerated due numerous Sertoli cells and widening of the lumen with vacuolated cytoplasm and dark nuclei and testicular parenchyma.

The basal membrane of the tubules was seen surrounded by spindle shaped cells.

Interstitial space in-between the tubules contain clusters of Leydig cells (LC) having vesticular nucleus with prominent nucleolus surrounded by congested blood vessel. The spermatogonia rest on basal membrane with round spermatid with widening of interstitial space containing cluster of leydig cells were observed in the inter-tubular space that was in closed

contact with blood vessels. The spermatogonia, Sertoli cells and the spermatid were markedly reduced in number with vacuolated cytoplasm and dark nuclei and testicular parenchyma appeared parked with tubules with extensive widening of lumen and interstitial space.

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