Full Length Research Paper

Short-term Scrotal Exposure to Elevated Temperature Prior to Mating Increase Male Ratio at Birth in Sprague-Dawley Rats

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ABSTRACT

This study was carried out to determine the effect of short-term exposure to elevated scrotal temperature at preconception on the testes and offspring sex ratio at birth. Ten adult Sprague-Dawley rats divided into two groups (N=5) were used. The scrota of the treatment group were immersed in a water bath regulated at 33 ± 2°C three times a day for 2 weeks. Control animals received same treatment in water of normal temperature (23 ± 2°C). After treatment, each male rat mated with a female rat on proestrous. Following conception, the male rats were sacrificed and testes harvested for histology, hormonal assay, seminal analysis, and antioxidant activities of catalase and superoxide dismutase. The pregnant dams were left to litter and foetal parameters and sex ratio were recorded. Male sex ratio increased in the treated group compared to control. Litter size, crown-rump length and tail length were reduced however; foetal weight was significantly higher in the treated group. Histology showed minimal disintegration of germ cell layers in few tubules and slight destruction of the interstitium and the interstitial cells. Reductions were observed in semen parameters (motility < 0.05), serum levels of FSH, LH, testosterone ($p < 0.05$) and antioxidant activities of superoxide dismutase and catalase in the treated group compared to the control. Male to female sex ratio increased at birth also, the short term exposure was able to attenuate the deleterious effect on semen parameters, histology and testicular weight, hormonal milieu and antioxidant status observed at long term exposure.

INTRODUCTION

Sex determination has been the subject of many human studies for a long time and it is a fundamental biological process in the development of individuals, leading to the formation of sex-ratios in natural populations. Literature is rife with the report of the deleterious effect of heat on testicular germ cells (MacLeod and Hotchkiss, 1941; Tunner *et al*., 2004; Bertolla *et al*., 2006; Paul *et al*., 2008; and Liu and Li 2010). Germ cells are more vulnerable to heat stress as they have high mitotic activity (Shiraishi *et al*., 2012). Studies have shown that among the germ cells, the types that are most vulnerable to heat are the pachytene and diplotene spermatocytes and the early round spermatids in humans (Carlsen *et al*., 2003) and rats (Chowdhury and Steinberger, 1970; Lue *et al*., 1999). It has also been reported that elevated testicular heat decreases sperm concentration, impairs motility, and reduces the number of morphologically normal sperms (MacLeod and Hotchkiss, 1941; Carlsen *et al*., 2003; Tunner *et al*., 2004) and these

parameters invariably impairs greatly on fertility. Offspring sex ratio at birth has been proposed to covary with ambient temperature. Studies by Lerchl (1999) and Helle *et al*., (2008) on humans have shown that proportionately more males were born during warm climates. Also in an animal study conducted by Hamid *et al*, (2012) in which pregnant female rats were exposed to elevated ambient temperatures more males than female litters were recorded.

We have reported earlier that exposure to elevated scrotal temperature of 33 ± 2°C three times a day for 4 weeks prior to mating increased male sex ratio at birth however, it produced marked deleterious effect on testicular cytoarchitecture, semen parameters, reproductive hormones, antioxidant status of superoxide dismutase and catalase in the testes (Gbotolorun *et al*., 2015).

This study was carried out to investigate whether a shorter duration of exposure will produce a similar desirable effect of increasing male to female sex ratio at birth and in addition, reduce the deleterious effects observed in the testes.

MATERIALS AND METHODS

Animals
A total of 10 male adult Sprague-Dawley rats weighing between 150 ± 30 g were used for this experiment. They were purchased from Peter's Farm Nigeria Enterprises, Badagry, Nigeria. The animals were housed in well-ventilated plastic cages under standard room temperature in the Animal House of the Department of Anatomy, College of Medicine of the University of Lagos. Rat feed and clean tap water were provided *ad libitum*. Two weeks of acclimatization to laboratory environmental conditions was provided before the commencement of the experiment. The animals were observed for adverse effects such as fur loss, diarrhea, bleeding, ataxia, morbidity and mortality resulting from administration of the extract. All procedures were approved by the Departmental Committee on the use and care of animals and tissue collection.

Experimental Groupings
The male rats were randomly selected and grouped into a test group and a control group with each group consisting of five animals each. The scrota of the male rats in the test group were immersed in a water bath at temperatures of 33 ± 2°C daily for 2 weeks. The immersion was performed 3 times daily at 07:00, 13:00 and 18:00 hours for duration of 5 minutes (Gbotolorun *et al.,* 2015). The control rats were immersed in a water bath of normal temperature at 23 ± 2°C.

Mating study
At the end of the treatment period, all the male rats were co-habited overnight with sexually mature females on proestrus phase of the oestrous cycle to encourage for mating at a ratio 1:1. Pregnancy was confirmed by the presence of spermatocytes in the vaginal smear. Immediately pregnancy was achieved the male rats were sacrificed while the pregnant dams were allowed to carry their pregnancy to full term and litter. Foetal parameters were recorded as crown-rump length, foetal size, tail length, foetal weight and sex ratio.

Sacrifice
The male rats were sacrificed by cervical dislocation. Blood was collected by cardiac puncture and stored at -80°C for hormonal assay studies on FSH, LH and Testosterone. The testes were removed; the left testes were assayed for antioxidant activities of superoxide dismutase and catalase while the right testes were fixed in Bouin's fluid for histology.

Seminal analysis
The caudal epididymides of the control and treated rats were excised and minced in 1 ml of normal saline and gently placed on clean slide. The sperm progressive motility was determined according to the method described by (Bearden and Fuquay, 1980). The sperm count was determined using the improved Neubauer haemocytometer (Deep 1/10 mm, LABART, GERMANY) as described by (Barratt, 1995; WHO, 1999). Microscopic examinations of the seminal smears stained with Eosin Nigrosin stain were carried out to determine the percentages of sperm morphology. This was done using a binocular microscope (Leica DM 750, SWITZERLAND) at 40x and 100x magnification.

Histology of the Testis
The fixed tissues were washed and dehydrated in ascending grades of alcohol. They were cleared in xylene, embedded in paraffin and sectioned at 5 µm thickness. Sections were placed on slides and stained with hematoxylin and eosin (H&E). Photomicrographs were made at magnifications of x100 and x400.

Hormonal Assay Studies on FSH, LH and Testosterone
Each blood sample was spun at 2,500 rpm for 10 minutes in an angle-head desktop centrifuge at temperatures of 25°C. Serum samples were assayed in batches with control sera at both physiological and pathological levels by Standard Quantitative Enzyme- Linked Immunosorbent Assay (ELISA) technique with Microwell kits from Syntro Bioresearch Inc., California, USA.

Testicular Homogenate for Antioxidant Activities of Superoxide Dismutase and Catalase
The left testes were homogenized using a Potter–Elvehjem homogenizer. A 20% (1/5 w/v) homogenate of the tissue was prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride and centrifuged at 10,000 rpm at 4°C for 10 min.

Superoxide dismutase was assayed utilizing the technique of (Kakkar *et al.,* 1984). A single unit of enzyme was expressed as 50% inhibition of Nitroblue tetrazo-lium (NBT) reduction/min/mg/protein.

Catalase was assayed colorimetrically at 620 nm and expressed as µmoles of H_2O_2 Consumed/min/mg/protein as described by (Sinha, 1972).

Statistical analysis
Results were expressed as Mean ± SEM (standard error of mean). Differences between groups were assessed using the student's t-test. *P* values less than 0.05 (*p*<0.05) was considered to be statistically significant.

RESULTS

No mortality was observed during the duration of the experiment. All the animals in the two groups appeared healthy and showed normal behaviour throughout the study.

Gestation length
Pregnancy was without any complications. There was no vaginal bleeding; pregnant dams appeared healthy and littered on day 20 post- conception. The length of gestation was not affected by the elevated scrotal temperature also, all the litters were viable at birth and no external congenital abnormalities were observed.

Effect of elevated scrotal temperature on foetal parameters and gender ratio
Crown-rump-length, tail-length and litter size of the foetuses in the treated group reduced compared to the control. However, a significant increase in foetal weight was observed

in the treated group. The litters were allowed to suckle for three weeks after which their genitals were examined. The male to female ratio in the control group was 4:11 while the male to female ratio in the treated group was 11:15 (Table 1).

Effect on testicular weight
Testicular weight decreased significantly in the treated group compared with the control as shown in table 2 below.

Table 1:
Effect of Elevated Scrotal Temperature on Foetal Parameters of Offspring and Gender Ratio in Sprague-Dawley Rats.

Treatment Groups	Gender Ratio		Crl	Tl	Fw	Ls
	Male	Female				
Control	1.60 ± 0.5	4.40 ± 0.5	4.80 ±0.1	1.74 ±0.2	5.24 ±0.2	6.00 ± 0.3
M/F Ratio	4	11				
Treated	2.20 ± 0.4	3.00 ± 0.3	4.37 ±0.1	1.51 ±0.0	5.57 ± 0.1*	5.40 ± 0.2
M/F Ratio	11	15				

Values represent mean ± S.E.M (N=5). *p< 0.05 vs. control.
CRL- crown rump length, TL- tail length, FW- foetal weight, LS- litter size, M/F- male/female

Table 2:
Effect of Elevated Scrotal Temperature on Testicular Weight Of Adult Male Sprague-Dawley Rats

Treatment groups	Testicular weight (g)
Control	1.04 ± 0.02
Treated	0.94 ± 0.04*

Values represent mean ± S.E.M (N=5). *p< 0.05 vs. control

Table 3:
Effect of Elevated Scrotal Temperature on Hormonal Milieu of Adult Male Sprague-Dawley Rats.

Treatment groups	Testosterone (ng/dL)	FSH (mlU/ml)	LH (mlU/ml)
Control	2.55 ± 0.1	0.51 ± 0.03	0.76 ± 0.04
Treated	1.34 ± 0.2*	0.31 ± 0.10	0.68 ± 0.05

Values represent mean ± S.E.M (N=5). *p< 0.05 vs. control.

Table 4:
Effect of elevated scrotal temperature on antioxidant activities of superoxide dismutase and catalase in adult male Sprague-Dawley rats.

Treatment groups	Superoxide dismutase (min./mg protein)	Catalase (Mmol/min./mg prot.)
Control	459.00 ± 63.6	2.45 ± 0.5
Treated	421.30 ± 40.0	1.96 ± 0.2

*Values represent mean ± S.E.M (N=5). *p< 0.05 vs. control.*

Effect on hormonal milieu
There was an observed decrease in serum levels of FSH, LH and testosterone ($p < 0.05$) in the treated groups compared to the control. This decrease was statistically significant for testosterone (Table 3).

Effect on oxidative stress markers
When the treated group was compared to control, there was a decrease in the activities of superoxide dismutase and catalase however, it was not statistically significant as observed in table 4.

Effect on seminal analysis
Sperm motility, sperm count and sperm morphology reduced in the temperature elevated group when compared to the control group. Sperm motility was statistically significant (Table 5).

Table 5:
Effect of elevated scrotal temperature on seminal analysis in adult Sprague-Dawley rats

| Seminal analysis	Control	Treated
Motility (%)	87.60 ± 4.70	54.60 ± 7.40*
Morphology (%)	84.60 ± 2.60	81.60 ± 3.10
Count (10^6/ml)	36.38 ± 5.80	27.04 ± 4.10

Values represent mean ± S.E.M (N=5). *p< 0.05 vs. control.

Effect on histology of the testes
Plates A and B showed the photomicrograph of testicular sections of a control rat demonstrating normal seminiferous tubules containing well organized arrangement of the cells of the spermatogenic series (ST). The spermatogonia cells (SC), sertoli cells (B), interstitial spaces and interstitial cells (IS) also appear normal as shown below. The lumen is seen filled with spermatids (A). Histological sections in the treated group showed disintegration of the cells of the spermatogenic series (ST) with slight widening and destruction of the interstitial spaces and cells (IS) (Plates C and D).

DISCUSSION

In this present study, histological sections of the testes showed disintegration of the spermatogenic series in a few of the tubules and a slight widening and destruction of the interstitium and interstitial cells. Germ cells are more vulnerable to heat stress as they have high mitotic activity (Shiraishi *et al.,* 2012) and the germ cell types most vulnerable to heat are the pachytene and diplotene spermatocytes and the early round spermatids (Chowdhury and Steinberger, 1970; Lue *et al.,* 1999). We have reported previously that exposing the scrotum to temperatures of 33 ± 2°C for four weeks caused a complete depletion/collapse of germ cell layers and the Sertoli cells in the seminiferous tubules with widening and destruction of the interstitial spaces and the interstitial cells (Gbotolorun *et al.,* 2015). One of the mechanisms by which germ cells incur damage is via apoptosis (Yin *et al.,* 1997; Lue *et al.,* 1999; Lue *et al.,* 2002).
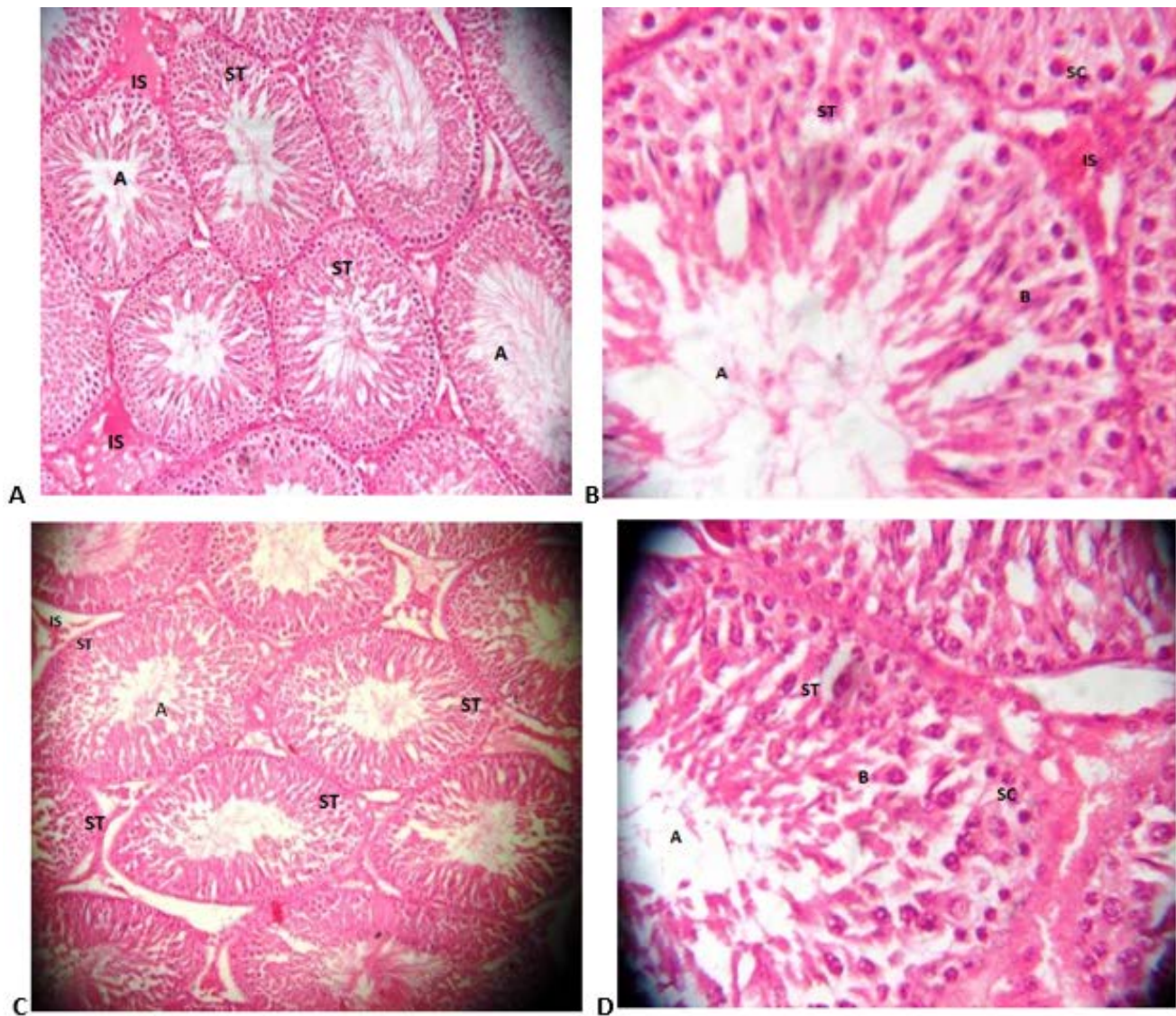
A. Light micrograph of the testes of the control, showing normal germinal epithelium with a well-organized arrangement of the cells of the spermatogenic series (ST), normal interstitium (IS) and lumen containing spermatids (A (H & E) ×100. **B**. Light micrograph of the testes of control group demonstrating normal Sertoli cells (B), and spermatogonia cell (SC). (H & E) ×400. **C**. Light micrograph of the testes of the treated group showing disintegration in the arrangement of the cells of the spermatogenic series (ST) and slight widening and destruction of the interstitium and intrerstitial cells (IS). (H & E) ×100. **D**. Light micrograph of the testes of treated rat showing disintegration in the arrangement of the cells of the spermatogenic series (B). (H & E) ×400.

In a study on the response of male germ cells to heat stress, Kim *et al,* (2013) reported that the apoptotic loss of germ cells is influenced by the severity and duration of heat stress that is, the higher the intensity or duration of exposure, the higher the apoptotic loss of germ cells. This may explain the reason why we observed an attenuation of the effect of heat on the cytoarchitecture of the testes in this study compared to the 4 weeks study.

In our previous report, a significant reduction was observed in sperm count, sperm motility and sperm morphology in the group exposed to elevated temperature for 4 weeks (Gbotolorun *et al.,* 2015). However, in this present study, of the 3 parameters studied, only sperm motility suffered significant reductions while slight reductions were observed in sperm count and sperm morphology when compared to the control. Elevated testicular heat has been reported to decrease sperm concentration, impair motility, and reduce the number of morphologically normal sperms (MacLeod and Hotchkiss, 1941; Carlsen *et al.,* 2003; Tunner *et al.,* 2004) this invariably, will impair on fertility and may likely explain the reduction in litter size observed in these studies.

This study recorded reduced serum levels of FSH, LH and testosterone ($p<0.05$) in the temperature elevated group compared to the control. It is a known fact that whereas FSH regulates spermatogenesis, LH controls Leydig cell function. Also, sperm production cannot proceed optimal to completion without continuous androgen supply. The report of this study is in concert with the report of Bughdadi (2014) who reported a reduction in the levels of FSH, LH and testosterone sequel

to testicular hyperthermia. A similar result was also recorded in our previous study of 4 weeks. Although the results of both the 2 and 4 weeks studies are similar however, the percentage reduction in the 2 and 4 weeks studies for testosterone (47.5 and 90.0), FSH (39.2 and 47.1) and LH (10.5 and 6.4) respectively, is indicative of the attenuating effect of a short-term exposure.

It is a known fact that testicular weight loss occurs subsequent to heat exposure (Sailer *et al.,* 1997; Setchell, 1998; Paul *et al.,* 2008; Durairajanayagam *et al.,* 2015). This present study showed that testicular weights in the animals exposed to elevated scrotal temperature were significantly reduced. Several studies have shown that testicular weight loss occurs subsequent to heat exposure (Sailer *et al.,* 1997; Setchell, 1998; Paul *et al.,* 2008; Durairajanayagam *et al.,* 2015; Gbotolorun *et al.,* 2015). Comparing the 2 and 4 weeks studies, it can be deduced that the 2 weeks study was able to attenuate the deleterious effect of heat on testicular weights as seen in the percentage reductions of 9.6 and 15.4 respectively. This is not unexpected because unlike the 2 weeks study, histological sections from the 4 weeks study had shown complete destruction and collapse of germ cell layers and Sertoli cells consequently, this may account for the higher reduction in testicular weight which may have likely occurred through depletions in hyperthermia-sensitive germ cells, mainly by apoptosis.

We reported a reduction in the antioxidant activities of superoxide dismutase and catalase in the animals exposed to elevated scrotal temperature for 4 weeks in our earlier study (Gbotolorun *et al.,* 2015). In this present study, we observed a similar trend of reduction in superoxide dismutase and catalase activities. The percentage reductions compared to the control for superoxide dismutase were 8.2 and 9.0 while catalase was 20.0 and 45.2 in both the 2 and 4 weeks studies respectively. From these results it can be inferred that the antioxidant activities of superoxide dismutase and catalase suffered the greater reduction in the 4 weeks study. Heat-stress increases oxygen radicals, possibly by the disruption of the electron transport assemblies of the membrane. Reactive oxygen species (ROS) have been reported to adversely affect spermatogenesis (Vicari and Calogero 2001). Heat-induced ROS formation is responsible for the molecular changes in DNA, proteins, lipids and other biological molecules that may contribute to low fertility (Lord-Fontaine and Averill-Bates 2002).

There is evidence that the X and Y spermatozoa are affected differentially by elevated temperatures. In the 4 weeks study, the treated group produced a male to female sex ratio of 1:1 (Gbotolorun *et al.,* 2015) while in the present study, the male to female sex ratio for the treated group was 11:15 while the control group was 4:11. It can be deduced therefore from the 2 weeks study that the male to female sex ratio increased markedly compared to the control. However, when the male to female sex ratio for the 2 and 4 weeks studies were compared, the 4 weeks study was higher than the 2 weeks. There is evidence that the X and Y spermatozoa are affected differentially by elevated temperatures. Although the results obtained from studies carried out so far by other researchers have been conflicting, however, our finding of a male-skewed sex ratio following exposure to elevated temperature supports previous reports suggesting that a warm temperature may increase sex ratios (Lerchl, 1999; Catalano *et al.,* 2008; Helle *et al.,* 2008; Hamid *et al.,* 2012).

In conclusion, short-term exposure to scrotal heat prior to conception increased the male to female sex ratio and also attenuated the deleterious effect of long-term exposure of heat on the testes. Howbeit, we recommend that antioxidants be administered concurrently with the heat treatment perhaps, the deleterious effect may be completely ruled out.

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