Original Article

Prolonged administration of proguanil induces reproductive toxicity in male rats

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ABSTRACT — The toxicity of proguanil was studied on reproductive activities in male rats using *in vivo* and *in vitro* methods. Groups of ten to twelve-week-old rats were administered proguanil (2.9 mg/kg body weight) daily for 5 days and 6 weeks respectively. Thereafter, body and reproductive organ weights were taken, sperm parameters were analyzed, while the histology of the testis and epididymis were carried out. Also, serum levels of testosterone, luteinizing hormone and follicle stimulating hormone were determined. Sertoli cells obtained from sixteen to eighteen day-old-rats were cultured and treated with 0.3 μ M to 10 μ M of proguanil for 5 days after which Sertoli cell viability and nuclei integrity were determined. Also, the genetic expressions of transferrin and Glial cell line-derived neurotrophic factor (G.D.N.F.) were assessed. The data obtained were analyzed using the analysis of variance (ANOVA) with Students-Newman-Keuls multiple comparison test. The results showed duration dependent significant decrease in body and organ weights and also in sperm parameters. Serum testosterone level was significantly decreased while luteinizing hormone and follicle stimulating hormone showed no significant change. Duration dependent degeneration was also observed in the testis and epididymis of treated rats. These changes were restored in recovery experiment. Viability and DNA integrity of treated Sertoli cells reduced in a dose and duration dependent manner. However, the genetic expressions of transferrin and Glial cell line-derived neurotrophic factor were normal. These results show that proguanil could induce infertility in rats which may act by distorting the blood-testis barrier formed by the Sertoli cells.

Key words: Proguanil, Sperm, Testosterone, Sertoli cells, Rat

INTRODUCTION

Malaria affects about 500 million people annually and is a leading cause of mortality and morbidity in developing areas of the world (Bremner *et al.*, 2004). It is an infectious disease caused by four species of *Plasmodium*; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. Of these, *P. falciparum* accounts for majority of clinical cases and mortalities (Bozdech *et al.*, 2003). This led to the development of antimalarial drugs to combat this scourge. Examples include quinine, chloroquine, pyrimethamine, sulphadoxine, proguanil and artemether. Proguanil is a type 2 antifolate inhibitor which inhibits dihydrofolate reductase (D.H.F.R.) (Reinaldo *et al.*, 2002). It is also prescribed as a prophylactic antimalarial drug (Prakash *et al.*, 2010).

Many antimalarial drugs have been associated with

male reproductive dysfunction. For example, chloroquine has been reported to reduce sperm motility and hence fertility by a reduction in the average number of fetuses of cohabited female rats (Adeeko and Dada, 1998). In another report, halofantrine adversely affected sperm parameters (Orisakwe *et al.*, 2003). The antifertility effect of pyrimethamine was also reported. It caused spermatogenic arrest and male infertility in a dose-dependent manner (Cosentino *et al.*, 1990). Artemether, an oral artemisinin derivative, caused a significant reduction in the progressive sperm motility, viability, sperm count and serum testosterone levels in a dose dependent manner during an acute administration of the drug in male rats (Raji *et al.*, 2005a). Interestingly, antimalarial medicinal plant extracts have also been implicated in experimental male infertility. For example, *Quassia amara* extract caused a significant reduction in testicular and epididymal weight,

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sperm count, motility and viability, serum levels of testosterone, luteinizing hormone and follicle stimulating hormone in male rats (Raji and Bolarinwa, 1997; Parveen *et al.*, 2003). *Azadirachta indica* extract was also reported to cause a mass atrophy of spermatogenic elements and Leydig cells in treated rats (Joshi *et al.*, 1996). In another study, the antifertility actions of *Morinda lucida* extract were reported. It caused a dose dependent decrease in sperm parameters when administered to male rats (Raji *et al.*, 2005b).

Proguanil has been reported to exhibit embryotoxicity. It was observed to be toxic at the cleavage of the ovum (Lyde, 1997). Fibrovascular proliferation in the right atrium, pyelonephritis, bone marrow hypocellularity, lymphoid atrophy, and gastritis/enteritis were observed in dogs treated with proguanil for 6 months at a dose of 12 mg/kg/day. Bile duct hyperplasia, gall bladder mucosal atrophy, and interstitial pneumonia were observed in dogs treated with proguanil for 6 months at a dose of 4 mg/kg/day. Mucosal hyperplasia of the cecum and renal tubular basophilia were observed in rats treated with proguanil hydrochloride for 6 months at a dose of 20 mg/kg/day. Adverse heart, lung, liver, and gall bladder effects observed in dogs and kidney effects observed in rats were not shown to be reversible. A fertility study in Sprague-Dawley rats revealed no adverse effects at doses up to 16 mg/kg/day. However, studies of proguanil in animals at exposures similar to or greater than those observed in humans have not been conducted (Malarone official FDA information, 2009). The global use of proguanil both as a therapeutic and prophylactic antimalarial drug thus led us to investigate its activities on male fertility.

MATERIALS AND METHODS

Drugs and Chemicals

Proguanil tablets (Paludrine) were produced by Zeneca Inc., Newark, DE, USA. The pure form of this drug was produced by and obtained from Unicure Remedies, Pvt Ltd. Baroda, India. The chemicals used in the Sertoli cell culture preparation were obtained from Sigma-Aldrich Inc, St. Louis, MO, USA.

Animals

Wistar strain albino rats (10-12 weeks; 180g-220 g) obtained from the Central Animal House, College of Medicine, University of Ibadan was used for the *in vivo* study while Sprague-Dawley albino male rats (16-18 days old; 18-22 g) obtained from the Central Animal House, Central Drug Research Institute, Lucknow, India was used for the *in vitro* study. The rats were provided with

feed and water *ad libitum* all through the period of the study. They were housed in wire mesh cages with a 12 hr light/12 hr dark cycle. The drug was administered to the rats by gavage. All the animals were cared for in accordance with the 'Guide for the Care and Use of Laboratory Animals' (1996). The use of these animals was reviewed and approved by the ethical committee at the Research Institute where the experiment was carried out.

Experimental Design

In vivo studies

Thirty male rats were used for this study and were divided equally into 5 groups. Group 1 contained control rats that were administered distilled water orally for 5 days. Group 2 contained rats administered 2.9 mg/kg B.W. of proguanil daily for 5 days. This was to mimic the dose and duration of administration of the drug in humans. Group 3 rats were given 2.9 mg/kg B.W. of proguanil daily for 6 weeks. This was to expose the gametes to the chronic effect of the drug (Heywood, 1983; Walker *et al.*, 1984). Rats in Group 4 received the same dose of proguanil daily for 6 weeks and were allowed to recover for 6 weeks. Group 5 rats are control rats that received distilled water for 6 weeks.

Anesthetic protocol and Autopsy

The rats were examined daily throughout the experimental period for signs of toxicity. At the end of the treatment and recovery period, they were killed by exsanguination under 25% urethane anesthesia (0.6 ml/100 g B.W.). Urethane has no known spermatogenic or antifertility effects on rat testis (Meneguz *et al.*, 1999). Body weight, weight of testes, epididymis and seminal vesicles were recorded at the time of necropsy.

Hormone assay

Blood was collected from the rats via cardiac puncture from which the serum was separated. Serum testosterone, luteinizing hormone and follicle stimulating hormone levels were measured in duplicate using the Enzyme-immunoassay (E.I.A.) technique (Raji *et al.*, 2005c). The E.I.A. kits were produced by Immunometrics (London, UK) and obtained from Nzemat (Lagos, Nigeria). The optical density was read using a spectrophotometer (Jenway, 6300 spectrophotometer, UK) that was sensitive at wavelengths between 492 nm and 550 nm.

Sperm characteristic analysis

The testes were carefully exposed and one of them was removed together with its epididymis. The epididymis was separated and epididymal fluid was collect-

ed from the caudal part. The progressive sperm motility, sperm count, live/dead ratio (viability) and morphology were determined as earlier described. (WHO, 1987; Farag *et al.*, 2000, 2007). Briefly, Progressive sperm motility was done immediately after semen collection. Two drops of semen were placed on the microscope slide and two drops of warm 2.9% sodium citrate were added. This was then covered with the cover slip and examined under the microscope using x40 objective with reduced light. Sperm viability was done using the eosin/nigrosin stain. The dead sperm took up the stain. Sperm morphology was carried out by means of the Walls and Ewas stain. Sperm count was carried out using the new improved Neuber's hemocytometer counting chamber.

Daily Sperm Production

Daily sperm production (DSP) was determined using a frozen testis by a procedure previously described (Robb *et al.*, 1978; Cooke *et al.*, 1992; Blazak *et al.*, 1993). Briefly, the testis was thawed at room temperature and homogenized in 1 ml of buffer solution containing 0.25 M sucrose and 0.02 M Tris (Sigma-Aldrich). The testis homogenate was diluted up to 5ml using the same buffer solution. Homogenization-resistant spermatids were counted using a Makler chamber (Sefi Medical Instrument, Haifa, Israel) under light microscopy.

Testicular and Epididymal histology

The testis and epididymis were prefixed in Bouin-Hollande solution prior to the histologic studies. They were then embedded in paraffin. Five microns thick paraffin sections were stained with hematoxylin-eosin stain and examined by light microscopy (Drury and Wallington, 1967; Akpantah *et al.*, 2003; Raji *et al.*, 2003).

Fertility Studies

Male rats treated for 6 weeks were cohabited with untreated parous proestrus female albino rats at ratio of 1:2. The presence of a vaginal plug was considered as an index of positive mating. A single time point fertility test for each rat was carried out using the formula: percentage fertility success is equal to the number of pregnant females divided by the mated females multiplied by 100. The number of litters delivered and their morphology was also recorded.

In vitro studies

Sertoli cells were obtained from the testis of 16-18 days old Sprague-Dawley rats (Kelly *et al.*, 1991) and cultured in DMEM/F-12 medium supplemented with insulin (5 mg/l), transferrin (5 mg/l), gentamicin sulfate (25

mg/l), vitamins A and E (200 ng/ml) and sodium bicarbonate (1.2 g/l) in a humidified atmosphere of 5% CO₂/95% air at 34°C at a density of 1 x 10⁶ cells/25 mm² area. After 48 hr, cells were treated with hypotonic solution (20 mM Tris buffer, pH 7.6) for 3 min to remove contaminating germ cells. The Sertoli cells were finally plated in 48-well tissue culture plates with fresh DMEM/F-12 medium supplemented further with testosterone (10⁻⁶ M) and follicle stimulating hormone (0.05 Units/ml).

The following procedures were then carried out on the cultured Sertoli cells;

Sertoli cell viability test (M.T.T. Assay)

The Sertoli cells were treated with 0.3 µM, 0.6 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM of the pure form of proguanil. The treatment was continued for 5 days *in-vitro* at 34°C in an atmosphere of 5% CO₂/95% air in a CO₂ incubator. After the duration of each treatment; 24 hr, 72 hr and 120 hr, 20 µl of M.T.T. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide] was added to each well of the 48-well tissue culture plates (Gong and Han, 2006). The solution was further incubated for 4 hr. Then the whole medium was removed from each well and replaced with 200 µl of Dimethyl Sulfoxide (D.M.S.O.) to dissolve the formazan crystals at 37°C for 30 min. The absorbance was then measured on an automated microplate reader (Microquant Biotech Instruments Inc.) at a wavelength of 540 nm.

Fluorescence microscopy of DAPI (4', 6-diamino-2-phenylindole) stained Sertoli cells

This procedure was used to determine the integrity of the double stranded deoxyribonucleic acid (dsDNA) of treated Sertoli cells. The cells were isolated (Kelly *et al.*, 1991) and plated on sterile cover slips in 12-well tissue culture plates at a density of 2.5 x 10⁶ cells/well and the cells were allowed to grow on these slips. The cells were then treated with 10 µM, 5 µM, and 2.5 µM of the pure forms of proguanil for five days. There was a positive control group which contained Sertoli cells that were treated only with Nonoxynol-9 (N-9), a known inducer of DNA fragmentation (Jain *et al.*, 2005). At the end of treatment period, the medium in each well was carefully removed. 500 µl of P.B.S. and 1 µl of 4'-6-Diamidino-2-phenylindole (DAPI) (from a stock solution of 1 µg/µl) was added to each well. This was allowed to incubate on a plate shaker for about one hour. The medium was then gently drained and the cover slips were then carefully removed from each well, mounted on slides and observed under the fluorescence microscope (Nikon Eclipse 80i) at an excitation wavelength of 350 nm. Apoptotic nuclei

were identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

Quantitative Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Sertoli cells were isolated (Kelly *et al.*, 1991) and plated into 6-well tissue culture plates at a density of 5×10^6 cells /well. The cells were then treated with 5 μ M, 2.5 μ M and 1.25 μ M of the pure forms of proguanil for five days. After the duration of treatment, the medium was removed and RNA was extracted from the treated cells. 2.5 μ g of each RNA sample was used to form complementary DNA using standard procedures (Muguruma *et al.*, 2005). The integrity of the house keeping gene, β -actin was checked and the expression of transferrin and GDNF (Glial cell line-derived neurotrophic factor) genes were determined using the Reverse Transcriptase Polymerase Chain Reaction technique and resolved using the electrophoresis technique on a horizontal agarose resolving gel assembly. The products were visualized by ethidium bromide (1 μ l of EtBr/10 ml of Agarose solution) staining in a gel duct (Boivis, Mumbai) under ultraviolet light. These genes are responsible for the proper growth of the Sertoli cells and essential for mammalian spermatogonial stem cell self-renewal. The rat gene primers (Sigma Genosys) used had the following forward and reverse sequences

β -actin gene

F = 5' AGGCATCCTGACCCTGAAGTA 3'

R = 3' TCTTCATGAGGTAGTCTGTCA 5'

Transferrin gene

F = 5' GCTGTGGCCAGTTTCTTCTC 3'

R = 3' CCACATCTCCACCTCCATCT 5'

GDNF (Glial cell line-derived neurotrophic factor) gene

F = 5' CCAATATGCCCGAAGATTATC 3'

R = 3' TTCGTAGCCCAACCCAAG 5'

Statistical Analysis

Data were presented as mean $\pm$ standard error ($X \pm$ S.E.) and statistical significance between control and treated groups was analyzed by the analysis of variance (ANOVA) with Students-Newman-Keuls multiple comparison test (Snedecor and Cochran, 1980) using the Graphpad Instat Statistical software package. Difference with probability value of $P < 0.05$ was considered significant.

RESULTS

Body and relative organ weights

There was no significant change in body and relative reproductive organ weights of rats administered proguanil (2.9 mg/kg B.W.) for five days except for the relative testicular weight which was significantly decreased ($P < 0.05$) when compared with the control (Table 1). There were significant reductions ($P < 0.01$) in all of these parameters in the rats treated for six weeks when compared with their control. However, a significant increase ($P < 0.01$) was observed in the recovery group when compared with the treated group (Table 2).

Effect of proguanil on sperm parameters

Administration of proguanil for five days significantly decreased ($P < 0.01$) the mean progressive sperm motility and viability of the treated rats when compared with the control. There was however no significant change ($P > 0.05$) in their mean sperm count (Table 3). All of these parameters were significantly decreased in rats treated daily for six weeks when compared with their control. Rats in the recovery group showed a significant increase ($P < 0.01$) in their sperm parameters when compared with the treated group (Table 4).

Administration of proguanil for 5 days caused no significant change ($P > 0.05$) in the number of abnormal sperms compared with the control (Table 5). There was however a significant increase ($P < 0.01$) in the number of abnormal sperms in rats treated for six weeks when compared with their control. Rats in the recovery group showed a significant decrease ($P < 0.05$) in the number of abnormal sperms when compared with the treated group (Table 6).

Effect of proguanil on daily sperm production in rats

Fig. 1 and Fig. 2 show the effect of proguanil administration on daily sperm production. There was a significant decrease ($P < 0.001$) in the daily sperm production of rats administered proguanil for five days when compared with the control (Fig. 1). This was also observed in rats treated for 6 weeks. However, rats in the recovery group showed a significant increase ($P < 0.001$) when compared with the treated group, with a gradual return of this parameter to control values (Fig. 2).

Effect of proguanil on some reproductive hormones

Administration of proguanil to rats for five days did not cause any significant change in serum LH and FSH

Induction of reproductive toxicity by proguanil treatment in male rats *in vivo* and *in vitro***Table 1.** Effect of proguanil on body and relative organ weights after 5 days treatment

Group	Body Weight (g)	Testes (%)	Epididymis (%)	Seminal Vesicle (%)
Control	200.00 ± 6.63	1.43 ± 0.02	0.20 ± 0.02	0.42 ± 0.02
Proguanil (5 days)	184.00 ± 4.17	1.33 ± 0.03*	0.18 ± 0.01	0.37 ± 0.05

Values are the mean ± S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control; *proguanil (5 days)* = *proguanil administration for 5 days*. * $P < 0.05$.

Table 2. Effect of proguanil on body and relative organ weights after 6 weeks treatment and recovery

Group	Body Weight (g)	Testes (%)	Epididymis (%)	Seminal Vesicle (%)
Control	286.00 ± 9.80	1.49 ± 0.03	0.29 ± 0.01	0.57 ± 0.03
Proguanil (6 weeks)	220.00 ± 9.70***	1.22 ± 0.04**	0.17 ± 0.01***	0.42 ± 0.02**
Recovery	272.00 ± 8.60**	1.33 ± 0.07	0.20 ± 0.01	0.53 ± 0.02**

Rats were treated for 6 weeks and then allowed to recover for 6 weeks. Values are the mean ± S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control while (•) indicates a significant difference between the treated groups and recovery; *proguanil (6wks)* = *proguanil administration for 6 weeks*; *recovery* = *recovery for 6 weeks*; ** $P < 0.01$, *** $P < 0.001$, ** $P < 0.01$.

Table 3. Effect of proguanil on sperm parameters after 5 days treatment

Group	Sperm motility (%)	Sperm viability (%)	Sperm count (10 ⁶ /ml)
Control	73.10 ± 1.30	73.60 ± 1.40	43.91 ± 1.82
Proguanil (5 days)	54.20 ± 1.36***	59.40 ± 1.39***	37.55 ± 1.40

Values are the mean ± S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control; *proguanil (5 days)* = *proguanil administration for 5 days*. *** $P < 0.001$.

Table 4. Effect of proguanil on sperm parameters after 6 weeks treatment and recovery

Group	Sperm motility (%)	Sperm viability (%)	Sperm count (10 ⁶ /ml)
Control	81.40 ± 1.86	85.80 ± 1.80	48.04 ± 0.92
Proguanil (6 weeks)	40.20 ± 2.81***	43.60 ± 3.01***	29.14 ± 0.96***
Recovery	74.80 ± 2.71***	79.80 ± 2.52***	40.49 ± 0.67***

Rats were treated for 6 weeks and then allowed to recover for 6 weeks. Values are the mean ± S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control while (•) indicates a significant difference between the treated groups and recovery; *proguanil (6wks)* = *proguanil administration for 6 weeks*; *recovery* = *recovery for 6 weeks*; *** $P < 0.001$, *** $P < 0.001$.

Table 5. Effect of proguanil on sperm morphological parameters after 5 days treatment

Group	% abnormal sperms
Control	8.67 ± 1.02
Proguanil (5 days)	12.44 ± 1.36

Values are the mean ± S.E. for groups of 6 rats.

Table 6. Effect of proguanil on sperm morphological parameters after 6 weeks treatment and recovery

Group	% abnormal sperms
Control	4.94 ± 0.63
Proguanil (6 weeks)	10.12 ± 1.27**
Recovery	6.67 ± 0.72•

Rats were treated for 6 weeks and then allowed to recover for 6 weeks. Values are the mean ± S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control while (•) indicates a significant difference between the treated groups and recovery; *proguanil (6wks)* = *proguanil administration for 6 weeks*; *recovery* = *recovery for 6 weeks*; ** $P < 0.01$, • $P < 0.05$.

Fig. 1. Effect of 5 days administration of proguanil on daily sperm production. Values are the mean $\pm$ S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control; * $P < 0.05$, *proguanil (5 days)* = *proguanil administration for 5 days*.

Fig. 3. Effect of 5 days administration of proguanil on serum LH and FSH concentration. Values are the mean $\pm$ S.E. for groups of 6 rats. *proguanil (5 days)* = *proguanil administration for 5 days*.

concentrations while there was a significant decrease ($P < 0.001$) in serum testosterone concentration when compared with the control (Figs. 3 and 4). This was also observed in rats treated daily for six weeks when compared with their control. There was a significant increase ($P < 0.001$) in serum testosterone level of rats in the recovery group when compared with the treated group, with a gradual return of this parameter to control values (Figs. 5 and 6).

Fig. 2. Effect of 6 weeks administration of proguanil on daily sperm production. Rats were treated for 6 weeks and then allowed to recover for 6 weeks. Values are the mean $\pm$ S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control while (*) indicates a significant difference between the treated groups and recovery; *proguanil (6wks)* = *proguanil administration for 6 weeks*; *recovery* = *recovery for 6 weeks*; *** $P < 0.001$, ** $P < 0.001$.

Fig. 4. Effect of 5 days administration of proguanil on serum testosterone concentration. Values are the mean $\pm$ S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control; ** $P < 0.01$, *proguanil (5 days)* = *proguanil administration for 5 days*.

Effect of proguanil on the histology of rat epididymis and testis

There were duration dependent histological changes observed in the epididymis and testis of the treated rats when compared with the control. Treatment for five days showed mild degenerative changes (Figs. 7 and 9) while those treated for six weeks showed severe degenerative changes (Figs. 8 and 10). There were visible reductions in

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Fig. 5. Effect of 6 weeks administration of proguanil on serum LH and FSH concentration. Rats were treated for 6 weeks and allowed to recover for 6 weeks. Values are the mean $\pm$ S.E. for groups of 6 rats. *proguanil (6wks)* = *proguanil administration for 6 weeks*.

Fig. 6. Effect of 6 weeks administration of proguanil on serum testosterone concentration. Rats were treated for 6 weeks and then allowed to recover for 6 weeks. Values are the mean $\pm$ S.E. for groups of 6 rats. Asterisks (*) indicates a significant difference between the treated group and control while (**) indicates a significant difference between the treated groups and recovery; *proguanil (6wks)* = *proguanil administration for 6 weeks*; *recovery* = *recovery for 6 weeks*; *** $P < 0.001$, *** $P < 0.001$.

Fig. 7. Effect of 5 days administration of proguanil on histology of rat epididymis. *Fig. 7a* shows the control epididymis while *Fig. 7b* shows the epididymis of rats administered proguanil daily for 5 days. Mag. $\times 100$ (ARROWS SHOWING STORED SPERM CELLS).

epididymal sperm content with vacuolization within the epididymis, while testicular histology showed disorganization of the seminiferous tubular architecture, degeneration of the plasmalemma, with arrest of spermatogenesis. However, there were regenerative and restorative changes observed in the epididymal and testicular histology of rats in the recovery group.

Effect of proguanil on litter size

There was a reduction in the number of pups produced by untreated female rats cohabited with treated male rats when compared with the control (Fig. 11).

Fig. 8. Effect of 6 weeks administration of proguanil on histology of rat epididymis. *Fig. 8a* shows the control epididymis; *Fig. 8b* shows the epididymis of rats administered proguanil daily for 6 weeks and *Fig. 8c* shows the epididymis of recovery rats. Mag. $\times 100$ (ARROWS SHOWING STORED SPERM CELLS).

Fig. 9. Effect of 5 days administration of proguanil on histology of rat testis. Fig. 9a shows the control while Fig. 9b shows the seminiferous tubules of rats administered proguanil daily for 5 days. Mag. x100 (ARROWS SHOWING SEMINIFEROUS TUBULES).

Effect of proguanil on mean viability of Sertoli cells *in vitro*

Fig. 12 shows the effect of proguanil on Sertoli cell viability. There was a dose and duration dependent decrease in the viability of the treated Sertoli cells. The lowest percentage of viable Sertoli cell was noted in cells treated with 10 μ M of proguanil for 120 hr while the highest percentage of viable Sertoli cells was observed in cells treated with 0.3 μ M for 24 hr.

Effect of proguanil on double-stranded Deoxyribonucleic acid (ds-DNA) integrity of Sertoli cells *in vitro*

There was also a dose and duration dependent fragmentation observed in the nuclei of Sertoli cells treated with the different doses of proguanil, with 10 μ M showing the greatest effect and 2.5 μ M showing the least at 120 hr (Figs. 13, 14 and 15).

Effect of proguanil on the expression of Glial cell line-Derived Neurotrophic Factor (GDNF) and Transferrin genes in Sertoli cells *in vitro*

Glial cell line-derived neurotrophic factor (GDNF) gene showed normal expression in cultured Sertoli cells treated with 5 μ M, 2.5 μ M and 1.25 μ M of proguanil when compared with the gene expression in control Sertoli cells. Similarly, transferrin gene expression in Sertoli cells treated with proguanil also showed normal expressions when compared with the control (Figs. 16 and 17).

DISCUSSION

We were able to report in this study that adminis-

Fig. 10. Effect of 6 weeks administration of proguanil on histology of rat epididymis. Fig. 10a shows the control seminiferous tubules; Fig. 10b shows the seminiferous tubules of rats administered proguanil daily for 6 weeks and Fig. 10c shows the seminiferous tubules of recovery rats. Mag. x 100 (ARROWS SHOWING SEMINIFEROUS TUBULES).

tration of proguanil by oral gavage caused significant adverse effects in the reproductive activities of the treated male rats especially at long term. Short term administration of proguanil had no negative effect on somatic growth except on testicular weight which was significantly reduced. However, there was a significant reduction in all of these parameters with prolonged administration of the drug. It has been reported that a change in relative or absolute weight of an organ after drug administration is an indication of the toxic effect of that drug (Simons *et al.*, 1995; Maina *et al.*, 2008). Also, the weight of male reproductive organs usually provides a useful fertility/reproductive risk assessment in experimental studies (Raji *et al.*, 2005c). Testicular size is the best primary assessment for spermatogenesis since the tubules and germinal elements account for approximately 98% of the testicular weight (Sherines and Howard, 1978). Thus the decrease observed in testicular weight can be linked to the degeneration of tubules and loss of germinal elements which was reported in this study. This finding is similar to an ear-

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Fig. 11. Effect of proguanil on litter size. Values are presented as mean $\pm$ S.E. *proguanil* = *proguanil* administration for 6 weeks.

Fig. 12. Effect of administration of proguanil on the viability of cultured Sertoli cells after 24 hr, 72 hr and 120 hr of treatment. Values are presented as mean $\pm$ S.E.

Fig. 13. Effect of 10 μ M proguanil on ds-DNA integrity of Sertoli cells. Fig. 13a shows the negative control (untreated Sertoli cells); Fig. 13b shows the positive control (N-9 treated Sertoli cells); Fig. 13c shows Sertoli cell nuclei treated with 10 μ M of proguanil for 24 hours; Fig. 13d shows Sertoli cell nuclei treated with 10 μ M of proguanil for 72 hours and Fig. 13e shows Sertoli cell nuclei treated with 10 μ M of proguanil for 120 hours. (ARROWS SHOWING SERTOLI CELL NUCLEUS) (Mag. $\times$ 40).

Fig. 14. Effect of 5 μ M proguanil on ds-DNA integrity of Sertoli cells. Fig. 14a shows the negative control (untreated Sertoli cells); Fig. 14b shows the positive control (N-9 treated Sertoli cells); Fig. 14c shows Sertoli cell nuclei treated with 5 μ M of proguanil for 24 hr; Fig. 14d shows Sertoli cell nuclei treated with 5 μ M of proguanil for 72 hr and Fig. 14e shows Sertoli cell nuclei treated with 5 μ M of proguanil for 120 hr. (ARROWS SHOWING SERTOLI CELL NUCLEUS) (Mag. $\times$ 40).

Fig. 15. Effect of 2.5µM proguanil on ds-DNA integrity of Sertoli cells. Fig. 15a shows the negative control (untreated Sertoli cells); Fig. 15b shows the positive control (N-9 treated Sertoli cells); Fig. 15c shows Sertoli cell nuclei treated with 2.5 µM of proguanil for 24 hr; Fig. 15d shows Sertoli cell nuclei treated with 2.5 µM of proguanil for 72 hr and Fig. 15e shows Sertoli cell nuclei treated with 2.5 µM of proguanil for 120 hr. (ARROWS SHOWING SERTOLI CELL NUCLEUS) (Mag. x 40).

lier one which reported that administration of artemether (Raji *et al.*, 2005a) and chloroquine (Okanlawon and Ashiru, 1998) significantly reduced the body and organ weights of the treated rats.

The progressive sperm motility and viability of rats treated with proguanil were significantly reduced at both the short and long term treatments. It is known that sperm cells gain their motility as they move through the different regions of the epididymis. As the spermatozoa moves through the corpus epididymis, motility increases sharply and continues to improve through the caudal epididymis and vas deferens (Horst *et al.*, 1999). These effects on motility and viability observed in this study have also been reported (Adeeko and Dada, 1998) in which chloroquine inhibited epididymal sperm motility by accumulating in the epididymis and interfering with the acquisition of motility. Cosentino *et al.* (1990) in their experiment at developing a male contraceptive reported that pyrimeth-

Fig. 16. Transferrin gene expression in cultured Sertoli cells. A shows the genetic expression in Sertoli cells treated with 5µM of proguanil; B shows the genetic expression in Sertoli cells treated with 2.5 µM of proguanil; C shows the genetic expression in Sertoli cells treated with 1.25 µM of proguanil; D shows the genetic expression in control Sertoli cells.

Fig. 17. GDNF gene expression in cultured Sertoli cells. A shows the genetic expression in Sertoli cells treated with 5µM of proguanil; B shows the genetic expression in Sertoli cells treated with 2.5 µM of proguanil; C shows the genetic expression in Sertoli cells treated with 1.25 µM of proguanil; D shows the genetic expression in control Sertoli cells.

amine caused infertility actions in the treated mice. The number of abnormal sperms observed in rats administered proguanil was not significant at short term administration but was significant with long term administration. This result was similar to that caused by administering sulphamethoxypyridazine;pyrimethamine (Metakelfin) to mice (Otubanjo and Mosuro, 2007). Short term administration of proguanil caused no significant change in daily sperm production but significantly decreased epididymal sperm count. However, long term administration significantly decreased both parameters. This could be linked with the testicular and epididymal damage reported in this study. In a similar finding, chlorpyrifos was reported to cause severe testicular damage which resulted in reduction in sperm count and thus fertility in male mice (Frag *et al.*, 2010; Joshi *et al.*, 2007).

Spermatogenesis is influenced and achieved by interplay of reproductive hormones secreted from the hypothalamo-pituitary-testicular axis relating gonadotrophin releasing hormone, luteinizing hormone, follicle stimulating hormone and testosterone. In this study, the concentrations of luteinizing hormone and follicle stimulating hormone in rats treated with proguanil both at short term and long term showed no significant change. Fol-

luteal stimulating hormone is known to stimulate mitotic division and proliferation of Sertoli and germ cells (Almiron and Chemes, 1988). The increase observed in follicle stimulating hormone concentration could therefore be ascribed to the feedback on the hormone due to loss of germ and Sertoli cells caused by long term administration of proguanil. Spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone (Farag *et al.*, 2010). In this study, serum testosterone concentration was significantly decreased both at short and long term administration. This could be due to testicular degeneration reported in this study, thus causing an arrest of spermatogenesis, reduction in sperm count and hence decrease in fertility which was evidenced by a reduction in litter size. A similar finding was also reported by Adeeko and Dada on their experiment with chloroquine (Adeeko and Dada, 1998). Histological examination revealed that proguanil caused moderate and severe testicular and epididymal lesions at both short and long term administration. Testicular histology showed degeneration of the plasmalemma with vacuolization of the seminiferous tubules. This might imply that these drugs were able to permeate the blood-testis barrier and caused arrest of spermatogenesis (Baldessarini, 1980). Histology of the epididymis showed a visible reduction in the sperm content. This could explain the decrease observed in sperm count of rats treated with proguanil. However, the histological section of the testis and epididymis of rats in the recovery groups showed regeneration of the germinal epithelium and germ cells, re-building of the different layers within the seminiferous tubules of the testis and thus re-establishment of spermatogenesis. These results were similar to published reports that artemether (Raji *et al.*, 2005a) and chloroquine (Okanlawon and Ashiru, 1998; Asuquo and Igri, 2007) caused degenerative effects in the testicular histology of treated rats.

However, in the recovery experiments, withdrawal of drug administration for 6 weeks resulted in recovery from the deleterious effects induced by proguanil. This infers that the toxic effects were not permanent but were reversible when the drug was withdrawn.

Sertoli cells are the somatic cells of the testis that are essential for spermatogenesis. They facilitate the progression of sperm cells to spermatozoa (Griswold, 1998). The results obtained from Sertoli cell viability study showed a dose and duration dependent decrease in number of viable Sertoli cells treated with proguanil *in vitro*. A reduction in the number of viable Sertoli cells due to drug toxicity will definitely expose the germ cells and the developing spermatocytes and spermatids within the seminiferous tubules of the testis to the drug's toxic effects. Sertoli cells also

constitute the blood-testis barrier (Buzzard, 2002). Thus damage to Sertoli cells may lead to impairment of male reproduction. This could explain why proguanil was probably able to permeate the blood-testis barrier and thus affect spermatogenesis as evidenced by the degeneration in testicular histological studies and also the reduction observed in the sperm count in this study. The molecular basis of Sertoli cell damage by proguanil in this study showed a dose and duration dependent degeneration on the double stranded deoxyribonucleic acid (ds-DNA) of Sertoli cells of the treated rats. It was observed that the greatest occurrence of nuclear fragmentation and chromatin condensation occurred in Sertoli cells treated with 10 μ M of proguanil for 120 hr. A similar effect was seen in Nonylphenol-9 treated Sertoli cells which served as the positive control. Nonylphenol-9 has been shown to induce adverse oxidative stress in rat Sertoli cells (Gong and Han, 2006). Based on the results obtained from the determination of the ds-DNA integrity, the expressions of two Sertoli cell genes were thus determined in this study. These genes were transferrin and Glial cell line-derived neurotrophic factor (G.D.N.F.). Transferrin gene codes the formation of the iron transport glycoprotein, transferrin and it is important in the delivery of iron to germinal cells within the adluminal compartment of the seminiferous tubules (Skinner and Griswold, 1982; Sylvester, 1993). GDNF is a strong neurotrophic factor which is expressed in the testis. It plays an important role in the proliferation of Sertoli cells (Hu *et al.*, 1999). The results obtained showed normal DNA expression of both transferrin and GDNF genes of the treated Sertoli cells at all the different doses used in this study. This shows that although proguanil caused some nuclear fragmentation and chromatin condensation, the DNA expressions of transferrin and GDNF genes remained unchanged at various tested doses.

Results of both the *in vivo* and *in vitro* studies showed that proguanil caused reversible adverse effects on male reproductive functions in a dose and duration dependent manner. The study of biological phenomena *in-vivo* is often complicated by various interactions operative within a living organism. Tissue culture models although highly artificial, provide valuable systems in which the environmental conditions can be controlled and the effect of various factors on a specific cell type can be investigated directly. However, morphological and functional characteristics are frequently subject to alteration in culture due to changes in pH, temperature, culture medium and atmospheric conditions, but care was taken to maintain these factors at physiological conditions suitable to the cultured cells in this study. The present *in-vitro* studies were therefore supported at least and in part by the

in-vivo experiments. Also, the long term use of proguanil might have to be done with caution because the greatest adverse effect was observed during the long-term administration of the drug. Further studies could be aimed at co-culturing Sertoli cells with germ cells and then treating them with proguanil. This will shed more light as to how these drugs affect the protective function of Sertoli cells on germ cells.

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