
# International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 2 (1): 9-14, 2006
ISSN 1811-7775


# *In vivo* and *in vitro* Reproductive Toxicity Assessment of Ampicillin and Cloxacillin in Mammalian Models

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**Abstract:** This study was carried out to investigate the individual impact of ampicillin and cloxacillin on male reproduction using both *in vivo* and *in vitro* models. In the *in vivo* study, forty adult male albino rats divided into five groups were treated daily with 0.5 mL sterile water (control), 4 mg/100 g b.w/day of ampicillin and 6 mg/100 g b.w/day of cloxacillin. Each drug treated group had a corresponding recovery group. Vehicle and drugs were administered orally for two weeks at the end of which rats were sacrificed; the recovery rats were sacrificed two weeks later. Body and reproductive organ weights and histomorphometric analyses of the testes and epididymides were carried out. Sperm counts, motility, viability and morphology and serum testosterone levels were determined. In the *in vitro* study, semen from the West African Dwarf Buck (WADB) was extended in graded concentrations (2.5, 5.0, 7.5, 10.0, 15.0 and 20.0 mg $mL^{-1}$) of ampicillin and cloxacillin individually for 24, 48, 72, 96 and 120 h. The *in vivo* results show that both drugs did not adversely affect body weigh but caused significant reduction ($p<0.05$) in the weight of the testes, epididymides, seminal vesicles and prostate glands. Similarly there was a significant decrease in sperm counts, motility, viability and morphologically normal spermatozoa. Seminiferous tubular diameter and epididymal ductular diameter were significantly reduced ($p<0.05$) in both ampicillin and cloxacillin treated rats when compared with the control. These changes were accompanied by significant decrease in serum testosterone levels. Discontinuation of treatment led to recovery of organ weighs, testosterone secretion and sperm functions. In the *in vitro* experiments, sperm motility was significantly reduced ($p<0.05$) in the two drugs. This reduction was both dose and duration dependent. The results suggest that ampicillin and cloxacillin could induce reversible infertility in male, which could be mediated by decrease in testosterone secretion.

**Key words:** Penicillin, sperm, testosterone, male, fertility

## INTRODUCTION

Humans are showing declining fertility. Many factors, including the increased use of antibiotics have been implicated as potential causes of male infertility. About 50% of known causes of primary infertility are now attributed to male factor[1]; however the aetiology of male factor is poorly understood. While certain individuals may be genetically predisposed to being subfertile[2], there are many epigenetic factors, which have been implicated as potential causes of male infertility. The male reproductive system is known to be highly sensitive to many environment pollutants[3]. Drugs and chemicals being part of the environment have been found to pose adverse effects on male reproductive capability under certain conditions[4].

Adverse effects of many antibiotics on male reproductive functions have been reported and reviewed elsewhere[5]. In man, these effects have been clearly delineated for a few agents and have been implicated for most classes of antibiotics. Nitrofurans[6], macrolides such as erythromycin and tylosin[7], aminoglycosides such as gentamicin and neomycin[8,9], tetracyclines[10] and sulfa drugs such as penicillins[8] have all been reported to cause varying degrees of spermatogenic inhibition in man and animal models. Reported reproductive effects include spermatogenic arrest, inhibition of testicular cell carbohydrate metabolism and oxygen consumption, impaired sperm motility and spermicidal activities.

Ampicillin has been shown to cause a significant decrease in fertilizing capacity and motility of chicken spermatozoa[11]. Similarly dicloxacillin had been reported to

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decrease sperm motility in bovine[12]. However, these observations have not been investigated in detail in mammalian species for possible extrapolation in humans. It is important to note that ampicillin and cloxacillin are broad-spectrum antibiotics that are used in the treatment of various bacteria infections. These drugs belong to the group of penicillin antibiotics. Penicillins are also often widely abused especially in underdeveloped and developing countries. Toxicological, hormonal and histological tests were carried out on male reproductive functions in albino rats and semen collected from West African Dwarf Buck (WADB) to evaluate the actions of ampicillin and cloxacillin in male reproduction.

## MATERIALS AND METHODS

**Animals:** Adult male albino rats of Wistar strain weighing between 200-240 g were used in this study. The rats were obtained from Central Animal House, College of Medicine, University of Ibadan and acclimatized to laboratory environment for one week before experimentation. The study was conducted between February and October 2001.

**Drugs and chemicals:** Smith Kline Beecham International Medicine manufactured the ampicillin, while cloxacilline was by Maxheal Pharmaceuticals India. These drugs were purchased from the University of Ibadan Health Centre. The enzyme-based immunoassay (EIA) kit was obtained from Immunometrics (UK, Ltd.) and contained testosterone EIA enzyme label, testosterone EIA substrate reagent and EIA quality control sample.

### *In vivo* studies

**Experimental design:** Forty male albino rats were housed in groups of eight rats per wire mesh cage and provided with rat cubes and water *ad libitum* throughout the period of the study. The animals were divided into five equal groups and treated as follows: Group I: Control, received 0.5 mL sterile water (vehicle for the drugs); Group II received 4 mg/100 g b.w ampicillin; Group III received 4 mg/100 g b.w ampicillin (recovery); Group IV received 6 mg/100 g b.w cloxacillin while Group V rats received 6 mg/100 g b.w cloxacillin (recovery). A suspension was made for each antibiotic using sterile water in concentration such that the daily dosage of the antibiotic was contained in 0.5 mL of the suspension. Vehicle and drug administration was done daily for two weeks using oral dosing needle, in all groups. Group III and V rats were allowed 2 weeks recovery period during which the drugs were not administered. All animals were weighed at the beginning of the experiment and at autopsy. They were also allowed free access to drinking water and feed *ad libitum*.

**Serum collection and testosterone assay:** Twenty- four hours after the last dosing, blood was withdrawn and the animals killed. Blood samples were collected into sterile bottles via cardiac puncture. Serum was separated from the blood and stored at –20°C for testosterone assay. The assay was done using the enzyme-based immunoassay method (ElA). A quality control sample was carried out at the beginning and at the end of the assay to ascertain acceptability with respect to bias and within-assay variation. The EIA kit used had a sensitivity level of about 0.3 nmol $L^{-1}$ (0.1 g $mL^{-1}$) of testosterone. The intra and inter-assay variations were 10.0 and 10.12%, respectively.

**Reproductive organ weights:** The tissues of the male reproductive system were excised from the rats, cleared of adherent fat and weighed immediately to the nearest milligram. The testes and epididymides were preserved for histological analysis.

**Semen collection and analysis:** Sperm characteristics analysis was done as previously described[13]. Semen sample was collected from the cauda epididymis onto a pre warmed microscope glass slide (27°C) for motility, viability and morphological studies. Sperm morphology was done by staining the sperm smears on microscope slides with two drops of Walls and Ewas stain and air-dried. The abnormal sperm were counted and the percentage calculated. Sperm counts were done using the improved Neubauer haemocytometer and the results expressed as count X $10^6$ $mL^{-1}$. The primary and secondary abnormalities in spermatozoa were counted, scored and expressed in percentage after staining with Nigrosin/eosin in 10:4 ratio.

**Histomorphometric studies:** Histological sections of the testes and epididymides of rats from all groups were carried out as previously described[13]. Briefly, the testes and epididymides from each rat were separately fixed in 10% formalin. A thin section (5 μm) of the tissue was made and finally stained with haematoxylin and eosin dye. Each slide was clean-blotted and mounted in Canada balsam under a cover slip. The dimensions of the Seminiferous Tubular Diameter (STD), Epididymal Luminal Diameter (ELD), Epididymal Ductular Diameter (EDD) and Epididymal Epithelial Height (EEH) were determined.

### *In vitro* studies

**Animal:** One healthy adult (3 years old) male West African Dwarf Buck (WADB) (*Carpora hircus* L.)

Table 1: Physiological characteristic of semen sample collected from the WADB

| Semen characteristics | Values |
|---|---|
| Ejaculate volume (mL) | 0.44 |
| Ejaculate colour | White creamy |
| Progressive motility (%) | 95.00±0.01 |
| Percentage life spermatozoa | 97.00±0.001 |
| Semen Concentration (x10 $mL^{-1}$) | 2.62 |
| Total sperm cell (x10 $mL^{-1}$) | 1.28 |
| pH | 6.7 |

weighing 20 kg was used for the *in vitro* study for ease of semen collection which was done using electro ejaculation method[14]. The animal was certified healthy and fertile by a veterinary doctor and was kept away from mating for 2 days prior to the study.

**Collection of semen samples:** Semen was collected from the goat into a pre-warmed (27°C) glass tube using electro-ejaculation-stimulating machine with a rectal probe. The semen quality (sperm count, 95.00±0.01 motility, 6.7 pH, 0.44 mL volume, white creamy colour and 97.00±0.001 percentage life spermatozoa) was quickly assessed (Table 1). It was thereafter extended in individual concentration of ampicillin and cloxacillin previously prepared and stored at 5°C.

**Preparation of extenders:** Extenders are liquid substances used when preserving semen samples for long period to allow the spermatozoa retain their functions, remain alive, provide nutrients and also to prevent growth of harmful bacteria. In this study extenders were prepared prior to semen collection. Extender 1 was the control extender and contained 90 mL of citrate buffer (2.9% w/v), 10 mL of egg yolk[12,15]. The pH of the extenders was maintained at 7.43 by means of buffers and kept in water bath at 37°C. Extender 2 (test extender) was prepared by diluting a stock solution of ampicillin (50 mg $mL^{-1}$) to give graded concentrations of 2.5, 5, 7.5, 10, 15 and 20 mg $mL^{-1}$. One milliliter each from the doses was added to 10 mL of extender 1. Extender 3 (test extender) was prepared by diluting a stock solution of cloxacillin (50 mg $mL^{-1}$) to give graded concentrations of 2.5, 5, 7.5, 10, 15 and 20 mg $mL^{-1}$. One milliliter each from the doses was added to 10 mL of extender 1.

**Progressive sperm motility study:** The progressive motility of the extended semen sample of graded concentration of ampicillin and cloxacillin (2.5-20.0 mg $mL^{-1}$) was done at an interval of 24 h until no motility could be observed.

**Statistical analysis:** Data were presented as mean±SEM. Statistical significance was determined using Student's t-test and ANOVA.

## RESULTS

### *In vivo* study

**Individual effects of ampicillin and cloxacillin on relative weight of reproductive organ:** All rats recorded about 8-10% weight gain within the period of the experiment. There was a significant decrease ($p<0.05$) in the weight of the testis, epididymis, seminal vesicle and prostate gland in all ampicillin and cloxacillin treated rats. A significant increase (improvement; $p<0.05$) in epididymal weight was however recorded in the recovery group of cloxacillin. There was no change in liver weight when compared with the control (Table 2).

**Individual effects of ampicillin and cloxacillin on spermatozoal functions in rats:** There was no significant change in the percentage life spermatozoa of drug treated and the recovery groups. However, a significant reduction ($p<0.05$) in percentage sperm motility was recorded in ampicillin and cloxacillin treated rats when compared with the control values. A significant increase (improvement; $p<0.05$) in these parameters was however recorded in the recovery groups (Table 3). The results of sperm morphology showed a mild primary abnormality in all the groups of ampicillin and cloxacillin treated rats. Most of the abnormalities recorded were of secondary forms. There was a significant reduction ($p<0.05$) in epididymal sperm counts in both ampicillin and cloxacillin treated rats (Table 3-5).

**Individual effects of ampicillin and cloxacillin on serum testosterone levels:** The mean serum testosterone level of control rats was 4.67±0.29 nM $L^{-1}$. This was significantly reduced ($p<0.05$) in all ampicillin and cloxacillin treated rats when compared with the control. However a significant restoration ($p<0.05$) in serum testosterone level towards that of the control group was recorded in the recovery groups (Table 6).

**Individual effects of ampicillin and cloxacillin on testicular and epididymal histomorphometry:** Dimensional studies of the STD revealed a reduction in STD in ampicillin and cloxacillin treated rats. There was a significant recovery in these parameters when compared with the control (Table 7). The ELD was not significantly affected in the cloxacillin group while it was reduced in all ampicillin test groups. ELD values returned toward normal control values within the period of recovery. Similar results were recorded for EDD as well as EEH in all ampicillin and cloxacillin treated rats. Epididymal dimensions were fully restored within the period of

Table 2: Individual effects of ampicillin and cloxacillin on relative organ weight in male albino rats. Values are presented as percentage organ weight per body weight

| Treatment groups (N=8) | Control | Ampicillin (4 mg/100 g/day) | Recovery | Cloxacillin (6 mg/100 g/day) | Recovery |
|---|---|---|---|---|---|
| Final Body weight (g) | 242.50±5.48 | 250.00±3.65 | 251.67±4.20 | 230.00±0.00 | 230.00±7.30 |
| Liver | 3.39±0.46 | 3.54±0.56 | 3.20±0.12 | 3.16±0.21 | 2.96±0.23 |
| Seminal vesicle | 0.38±0.04 | 0.09±0.02* | 0.30±0.02 | 0.14±0.01* | 0.40±0.03 |
| Prostate gland | 0.13±0.01 | 0.08±0.00* | 0.14±0.02 | 0.07±0.01* | 0.10±0.03 |
| Testis | 0.58±0.04 | 0.40±0.08* | 0.48±0.04 | 0.41±0.03* | 0.40±0.06 |
| Epididymis | 0.18±0.01 | 0.09±0.03* | 0.17±0.04 | 0.09±0.02* | 0.14±0.00 |

*Significantly different from control ($p<0.05$)

Table 3: Individual effects of ampicillin and cloxacillin on sperm motility and viability spermatozoa life-death ratio in male albino rats

| Treatment groups (N=8) | Control | Ampicillin (4 mg/100 g/day) | Recovery | Cloxacillin (6 mg/100 g/day) | Recovery |
|---|---|---|---|---|---|
| (%) Motility | 97.16±0.2 | 84.33±1.77* | 96.67±1.09 | 81.67±0.49 | 96.00±1.37 |
| (%) Viability | 98.00±0.02 | 98.00±0.00 | 98.00±0.00 | 98.00±0.20 | 98.00±0.00 |
| Life death ratio | 49:1 | 49:1 | 49:1 | 49:1 | 49:1 |

*Significantly different from control ($p<0.05$)

Table 4: Individual effects of ampicillin and cloxacillin on the morphology of rat spermatozoa *in vivo*

| Treatment groups (N=8) | (%) Primary abnormality | (%) Secondary abnormality | (%) Total abnormality |
|---|---|---|---|
| Control | 0.00 | 0.00 | 0.00 |
| Ampicillin (4 mg/100 g/day) | 2.37±0.13 | 31.64±0.66 | 34.18±0.71* |
| Ampicillin recovery | 0.00±0 | 2.17±0.20 | 2.17±0.20* |
| Cloxacillin (6 mg/100 g/day) | 5.16±0.82 | 29.25±0.91 | 33.67±0.93* |
| Cloxacillin recovery | 0.00±0 | 2.88±0.47 | 2.88±0.46* |

*Significantly different from control ($p<0.05$)

Table 5: Individual effects of ampicillin and cloxacillin on epididymal sperm count in albino rats

| Treatment groups (N=8) | Control | Ampicillin (4 mg/100 g/day) | Recovery | Cloxacillin (6 mg/100 g/day) | Recovery |
|---|---|---|---|---|---|
| Epididymal volume (mL) | 0.23±0.11 | 0.10±0.00* | 0.20±0.00 | 0.10±0.00* | 0.20±0.00 |
| Sperm count $X10^6$ $mL^{-1}$ | 65.67±11.73 | 33.83±5.63* | 64.40±25.78 | 36.17±3.04* | 64.40±12.78 |

*Significantly different from control ($p<0.05$)

Table 6: Individual effects of ampicillin and cloxacillin on serum testosterone level in male albino rats

| Treatment groups (N=8) | Control | Ampicillin (4 mg/100 g/day) | Recovery | Cloxacillin (6 mg/100 g/day) | Recovery |
|---|---|---|---|---|---|
| Serum testosterone level nM $L^{-1}$ | 4.67±0.29 | 0.34±0.05* | 4.40±0.71 | 0.30±0.08* | 4.48±0.82 |

*Significantly different from control ($p<0.05$)

Table 7: Individual effects of ampicillin and cloxacillin on seminiferous tubular diameter (STD), epididymal luminal diameter (ELD), Epididymal Ductular Diameter (EDD) and Epididymal Epithelia Height (EEH) in male albino rats

| Treatment group (N=8) | Seminiferous tubular diameter (mm) | Epididymal luminal diameter (mm) | Epididymal ductular diameter (mm) | Epididymal epithelial height (mm) |
|---|---|---|---|---|
| Control | 0.24±0.03 | 0.21±0.04 | 0.40±0.02 | 0.09±0.02 |
| Ampicillin (4 mg/100 g/day) | 0.17±0.08* | 0.15±0.05* | 0.33±0.10* | 0.06±0.01* |
| Ampicillin Recovery | 0.21±0.03 | 0.21±0.10 | 0.44±0.11 | 0.11±0.02 |
| Cloxacillin (6 mg/100 g/day) | 0.18±0.01* | 0.18±0.02* | 0.25±0.02* | 0.01±0.01* |
| Cloxacillin Recovery | 0.27±0.11 | 0.31±0.14 | 0.54±0.22 | 0.06±0.01* |

*Significantly different from control ($p<0.05$)

recovery. The reduction in epididymal dimensions was much more pronounced in cloxacillin groups than in the ampicillin groups.

### *In vitro* study

**Individual effects of ampicillin and cloxacillin on progressive sperm motility of WADB *in vitro*:** There was a significant reduction ($p<0.05$) in percentage sperm motility with increasing concentration of the drugs (2.5-20 mg $mL^{-1}$) and increasing time of extension in ampicillin and cloxacillin extenders. The maximum extension period in both ampicillin and cloxacillin was 96 h by which time the motility has reduced to 14% (at concentration of 2.5-5 mg $mL^{-1}$) and 7% (at concentration of 10 mg $mL^{-1}$) for each drug. All sperm were dead beyond this period of extension. There was however a significant reduction ($p<0.05$) in sperm motility (5.00±0.71%) when compared with the control (Table 8 and 9).

## DISCUSSION

In this study ampicillin and cloxacillin did not show adverse effects on body weights of rats. However, the epididymis, seminal vesicle and prostate glands showed significant reduction in weight in the drug treated rats. It is well established that changes in either absolute or relative weight of an organ following administration of

Table 8: Effects of ampicillin on progressive sperm motility in extended buck semen stored at 5°C

| Ampicillin | Extender 1 (EYC)* as Control | Extender 2 | | | | | |
|---|---|---|---|---|---|---|---|
| % Concentration of ampicillin mg $mL^{-1}$ | Nil | 2.5 | 5.0 | 7.5 | 10.0 | 15.0 | 20.0 |
| % Motility immediately after the addition | 70.00+0.00 | 70.00+0.00 | 70.00+0.00 | 70.00+0.00 | 70.00+0.00 | 70.00+0.00 | 70.00+0.00 |
| % Motility after | | | | | | | |
| 24 h | 60.00+1.41 | 60.00+0.71 | 60.00+0.00 | 60.00+0.71 | 50.00+0.00 | 40.00+1.41 | 40.00+0.71 |
| 48 h | 20.00+1.14 | 50.00+0.00 | 50.00+1.41 | 40.00+1.41 | 40.00+1.41 | 40.00+1.41 | 40.00+1.41 |
| 72 h | 10.00+0.00 | 40.00+1.41 | 40.00+0.00 | 20.00+0.00 | 10.00+0.71 | 5.00+0.71 | 5.00+0.71 |
| 96 h | Nil | 10.00+0.00 | 10.00+0.71 | 5.00+0.71 | 5.00+0.71 | Nil | Nil |
| 120 h | Nil | Nil | Nil | Nil | Nil | Nil | Nil |

*EYC: Egg yolk sodium citrate

Table 9: Effects of cloxacillin on progressive sperm motility in extended buck semen stored at 5°C

| Cloxacillin | Extender 1 (EYC) as Control | Extender 3 | | | | | |
|---|---|---|---|---|---|---|---|
| % Concentration of cloxacillin mg $mL^{-1}$ | Nil | 2.5 | 5.0 | 7.5 | 10.0 | 15.0 | 20.0 |
| % Motility after the addition of antibiotics | 70.00±0.00 | 70.00±0.00 | 70.00±0.00 | 70.00±0.00 | 70.00±0.00 | 70.00±0.00 | 70.00±0.00 |
| % Motility after (Immediately) | | | | | | | |
| 24 h | 50.00±0.71 | 50.00±1.41 | 50.00±0.41 | 30.00±0.71 | 30.00±1.71 | 30.00±1.41 | 30.00±0.71 |
| 48 h | 50.00±0.00 | 40.00±1.41 | 30.00±0.71 | 15.00±1.41 | 15.00±1.41 | 15.00±1.41 | 15.0±00.71 |
| 72 h | 40.00±1.41 | 30.00±0.00 | 30.00±1.41 | 30.00±0.71 | 10.00±0.00 | 10.00±1.41 | 10.00±0.00 |
| 96 h | 30.00±1.41 | 5.00±0.71 | 5.00±0.00 | 5.00±0.71 | 5.00±0.71 | 5.00±0.71 | 5.00±0.00 |
| 120 h | 10.00±0.00 | Nil | Nil | Nil | Nil | Nil | Nil |

a drug is an indicator of the toxic effect of the drug on the organ[16]. The observed decrease in weight of these organs indicates that ampicillin and cloxacillin might be toxic to them.

Antimicrobial therapy has been shown to significantly affect spermatogenesis and seminal parameters in both human and animal models. This has been demonstrated with the use of nitrofurantoin[17] and furacin[6,18]. Furacin had been used to treat testicular germ cell tumours in the past[18]. Sulfasalazine that is commonly used in the treatment of inflammatory bowel disease and has been demonstrated to cause oligospermia and poor sperm motility is well known to cause decreased seminal quality[19]. The derivative of the sulfa drug group and other members in the class has been shown to cause decreased sperm counts. Many other antibiotics have been demonstrated to cause a decrease in male fertility potential. Tetracycline derivatives, specifically tetracycline hydrochloride and others, have been shown to cause a decrease in the spermatogenic index[10]. Other drugs that have been studied with respect to male reproductive functions include the macrolide group, such as erythromycin, spiramycin and neomycin[8] and tylosin[20,21]. Penicillin-G, ampicillin and dicloxacillin (Penicillin group), have all been implicated in causing spermatogenic arrest[8]. Consequently, the findings in the present study mirror some of the reported activities of antibiotics in spermatogenesis

Accompanying the reduction in epididymal weight in ampicillin and cloxacillin treated rats in the present study were corresponding significant reduction in epididymal sperm count. This finding corroborates previous reports in human and animal subjects[19,22]. In addition an increase in abnormal spermatozoa recorded in all ampicillin and cloxacillin treated rats could probably be linked to alteration in the epididymal functions. Prolonged administration of ampicillin and cloxacillin at the doses employed in this study resulted in pathological changes of testicular and epididymal structure, which probably, led to the degeneration of spermatocytes and spermatogenic arrest in some of the tubules. Delayed spermiation is characteristically seen when testosterone production is impaired[23]. This may underlie the reduction in sperm count noticeable with the antibiotic treated rats since the spermatids failed to undergo spermiation into the epididymis.

The antibiotics studied also significantly reduced sperm motility and therefore could be toxic to the sperm as they accumulate in the epididymis, which corroborate earlier findings[22,24]. Varying degrees of spermatogenic arrest as revealed by tubular degeneration and coagulated necroses were recorded in both ampicillin and cloxacillin test groups. This was further supported by the reduction in STD recorded in all ampicillin and cloxacillin treated rats compared with the control rats. These changes could be induced by the significant decrease in testosterone secretion observed in this study, since the hormone is required for growth and maintenance of male reproductive organs[23]. The recovery was also confirmed as the STD values in both ampicillin and cloxacillin recovery group returned towards the values obtained for the control rats. A reduction in ELD and EEH was observed in all ampicillin but not cloxacillin treated rats. These dimensional changes are closely linked to the reduction in testosterone secretion, which was adversely affected.

The *in vitro* study also confirmed a dose dependent alteration of spermatozoa motility, which is very important for fertilization to be achieved. This result further revealed

that cloxacillin adversely affected sperm motility more than ampicillin. Similar findings have been reported earlier in an *in vitro* study on the effect of co-trimoxazole, erythromycin, amoxycillin and tetracycline on sperm functions[24]. The toxic nature of these drugs on spermatozoal function was very marked when the sperm motility was evaluated at the intervals of 24 h for 5 days (Table 8 and 9) using the semen extender 1 as control. The drugs arrested the unidirectional progressive movements shown by the reduction in motility as the dosage of the drugs was increased. It is therefore concluded that ampicillin and cloxacillin could adversely affect male fertility through impairment of sperm motility. It is suggested that the effects of antibiotics on sperm function are known when treating male patients who still desire fertility for an infection.

## ACKNOWLEDGMENTS

The authors are grateful to the Senate of the University of Ibadan for support through a Senate Research Grant SRG/COM/1996/2B.

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