Oxidative Stress-Induced Spermiotoxicity of Ciprofloxacin: An *In Vitro* Study

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Abstract

Infertility can primarily result from oxidative stress induced by the use of various drugs including known antibiotics. This study investigated dose and time-dependent effect of Ciprofloxacin on antioxidant status, sperm motility, viability and morphological characteristics of semen from sexually mature boar. Semen was incubated with Ciprofloxacin at 5.4 µg/ml, 54 µg/ml and 540 µg/ml for 4 hours at 37^{o}C and sperm characteristics were monitored at 2-hour intervals. Antioxidant status was assessed after the incubation period by estimation of reduced glutathione levels, and activities of glutathione-S-transferase, glutathione peroxidase, superoxide dismutase and catalase. The extent of lipid peroxidation and hydrogen peroxide generation were also determined. Ciprofloxacin induced oxidative stress in boar semen in a dose-dependent manner with a significant increase ($p < 0.05$) in reduced glutathione levels, and activities of glutathione-S-transferase, glutathione peroxidase, and catalase as well as malondialdehyde and hydrogen peroxide levels when compared with the control. However, when compared with the control, there was a significant ($p < 0.05$) dose-dependent decline in the activity of SOD. Assessment of sperm characteristics revealed a decline in motility, 2 hours post-treatment with all doses of Ciprofloxacin which became significant in the 4th hour. Semen incubation with Ciprofloxacin also increased the number of abnormal sperm cells while decreasing viability in a time-dependent manner. Overall, these results show that Ciprofloxacin caused enhancement of free radicals and alterations in activities of antioxidant enzymes with a resultant deterioration in sperm motility, viability and morphology.

Keywords: Ciprofloxacin, oxidative stress, antioxidant status, semen, sperm.

1. Introduction

Ciprofloxacin (CPFX) is a 4-fluoro-quinolone antibiotic belonging to the second-generation fluoroquinolones with a broader antibacterial spectrum of activity [1]. Unlike many quinolones, its broad spectrum of activity against most strains of gram-negative and certain gram-positive bacteria, especially *S. pneumonia*, confers on it a drug of choice used by fertility specialists and urologists to treat infections of the urinary tracts and reproductive tissues [2 - 4]. Although a high level of regression in the infectious pathologies of the male reproductive tract has been achieved with the administration of ciprofloxacin, patients treated exhibited reduced sperm motility and viability and compromised sperm morphology, without improvement in sperm agglutination and leukocytospermia [5 - 6]. This observation is supported by results from experimental studies that demonstrated significant Leydig cell degeneration, reduced testis weight, testosterone, (luteinizing hormone) LH, (follicle stimulating hormone) FSH and prolactin levels, and sperm quality in a dose and time-dependent manner [7 - 9].

The antibacterial mechanism of CPFX is based on the inhibition of the bacterial type II topoisomerase (DNA gyrase) and topoisomerase IV. This mechanism of action, attributed to its ability to acidify cell cytoplasm, may be related to mitochondrial damage thus impeding mitochondrial DNA replication [10]. CPFX has also been demonstrated to stimulate the production of reactive oxygen species (ROS) in bacterial cells as a core mechanism of action [11 - 13].

Several adverse effects reported with the use of CPFX includes its cross-reactivity with mammalian topoisomerase II [14], and cytotoxic effects of CPFX reveal that it inhibits the growth of various cultured mammalian cells [15-16]. Others include hepatotoxicity [17], nephrotoxicity [18], carcinogenicity, phototoxicity [16, 19], chondrotoxicity and tendinopathy, [20 - 21] e.t.c. Amongst other adverse effects reported, CPFX

impairs testicular and epididymal function and structure [20 - 21]. Besides this, the increase in ROS level during CPFX antibacterial action may contribute to its deleterious effects on testicular function [22]. It is thus becoming obvious that the toxic effects associated with CPFX may have a direct or indirect link with certain mechanisms associated with its bactericidal activity.

Following administration, high levels of this drug were detected in prostatic tissue and seminal fluid [23 - 25]. Decreased sperm count, motility, viability, DNA damage, chromatin and morphological abnormalities of sperm cells as well as fertilization rates and embryonic development have also been reported with CPFX treatment [8, 9, 24, 26]. The concern about its toxic effect on male reproductive function has triggered more recent investigations which seek to unravel possible mechanisms underlying this challenge. Most of these aim at proffering possible modulatory or ameliorating approaches to addressing its toxicity while still harnessing its therapeutic benefits. Thus, this study seeks to verify this claim in a condition of CPFX-induced spermiotoxiciy in boar semen.

2. Materials and Methods

2.1. Chemicals

Ciprofloxacin injection USP (2 mg/ml in a bottle of 100 ml Ciprotab I.V) was purchased from Danax supermarket and pharmacy, Dugbe, Ibadan. Reduced glutathione, 5, 5′ -dithio-bis-2-nitrobenzoic acid, thiobarbituric acid, 1-chloro-2,4-dinitrobenzene (CDNB) and epinephrine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade and the purest quality available.

2.2. Incubation Medium (Tris-citric-fructose (TCF) extender)

Semen incubation medium consisted of Tris-(hydroxymethyl)-aminomethane (37.85 g/l), citric acid anhydrous (21.15 g/l) and D (-) fructose (10 g/l), pH 7.0. [27].

2.3. Animals and Semen Collection

Twelve testes were obtained from the scrotum of six freshly slaughtered sexually matured boars (6-18 months) at the abattoir market in Ibadan and were immediately transferred to the laboratory in a well-insulated flask maintained at 37ºC. Animal handling was done in accordance with measures provided by the US National Academy of Science (NAS) published by the US National Institute of Health on the use and care of experimental animals. The approval of the veterinary consultant(s) committee of the Bodija abattoir on ethics and animal handling was also obtained. The right and left epididymides were trimmed off testes and semen was collected into the incubation medium as described by [28]. Briefly, semen was flushed into the incubation medium (kept at room temperature) with 2 - 3 drops of the same through a 1.0 cm incision made with a scalpel blade on the caudal epididymis. Prior to CPFX treatment, aliquots of spermatozoa from each epididymis were evaluated for concentration and motility under a light microscope. Percentage sperm motility (>70%) was chosen as normal and used in this study.

2.4. Ciprofloxacin Treatments

Three different concentrations of ciprofloxacin were used for this study: 5.4 µg/ml (physiologic concentration), 54 µg/ml (10 X physiologic concentration) and 540 µg/ml (pharmacologic concentration) [29]. CPFX at various concentrations were added to semen in an incubation medium at 37 ºC (Table 1). All experiments were repeated five times under the same conditions and samples were incubated for 4 hours. Motility, viability and morphology characteristics were evaluated [30, 31] at 0, 2 and 4 hours. Afterwards, samples were centrifuged and the supernatant was used for antioxidant assay.

Table 1: *In vitro* Treatment of Boar Semen with CPFX

Treatments	Semen in TCF medium (ml)	Pure TCF medium (ml)	CPFX (ml)
Control	5	5	-
CPFX (5.4 µg/ml)	5	4.973	0.027
CPFX (54 µg/ml)	5	4.73	0.27
CPFX (540 µg/ml)	5	2.3	2.7

TCF medium = incubation medium, CPFX = ciprofloxacin, stock solution of CPFX= 2 mg/ml; total test tube volume = 10 ml; treatment (ml) in 10 ml total volume = C1V1 = C2V2

2.5. Sperm motility, Morphological Abnormalities and Percentage Viability Assay

Progressive sperm motility (rapid forward movement) was assessed by the method described by [30] and expressed as percentages. An aliquot of sperm suspension was smeared out on a clean glass slide with another slide and stained with Wells and Awa's stain (0.2 g of eosin and 0.6 g of fast green dissolved in distilled water and ethanol in the ratio 2 : 1) for morphological examination and 1% eosin B and 5% nigrosine in 3% sodium citrate dehydrate solution for determination of sperm live/dead ratio (viability) according to the method described by [31].

2.6. Biochemical Assays

At the end of the incubation period, spermatozoa suspensions in the TCF medium were homogenized for 10 s and centrifuged at 2000 g for 10 min at 4 ^{0}C. The supernatant obtained was used to determine levels of reduced Glutathione (GSH) and the Lipid Peroxidation (LPO) product malondialdehyde [32 - 33]. Glutathione- S-transferase activity (GST) was assayed using the method of Habig *et al.* [34] while Glutathione Peroxidase activity (GPX) was determined by the method of Rotruck *et al* [35]. Superoxide Dismutase (SOD) and Catalase (CAT) activities were evaluated by the methods described by Misra and Fridovich [36] and Clairborne [37]. Hydrogen peroxide (H_2O_2) generation was quantified according to the method of Wolff [38].

2.7. Statistical Analysis

Data are mean ± SD; Student's T-test was used to analyse data. $p < 0.05$ was of statistical significance.

3. Results and Discussion

Several *in vivo* and *in vitro* studies reported the adverse effect of CPFX on male reproductive function [7, 24, 39, 40]. The claims on its mechanism of toxicity are still being researched and confirmed as CPFX remains among the first-line choice of antibiotics used to treat a wide range of bacterial infections. This is because of its wide spectrum of activity, favourable pharmacokinetics and pharmacodynamics [41] as well as its availability and affordability. Our study is the first to report that CPFX significantly impairs sperm motility and viability *in vitro* by compensatory elevations of endogenous antioxidants as a plausible response to increased ROS production. Although CPFX is used by urologists and fertility specialists particularly in *in vitro* fertilization (IVF) techniques and in treating infection-associated infertility in males [3, 4], long exposure to this drug may defeat the intending aim. High concentrations of this drug could be sustained in semen following administration. It was reported by Wagenlehner and Naber [42] that the median seminal fluid concentration and the median ratio of seminal/plasma drug concentration of CPFX after 2-4 hours in volunteers were 6.57 mg/l and 7.70 mg/l respectively after an oral dose of 750 mg. Thus, ciprofloxacin is concentrated several-fold in ejaculate and seminal fluid and although sufficiently elevated to include the total spectrum of sensitive strains causing bacterial prostatitis, could be the reason for its detrimental effect on the testis and healthy sperm cells as shown in previous studies [4, 7, 22, 43] and supported by our study. CPFX concentration ≥ 5.4 µg/ml (physiologic concentration) significantly decreased the percentage of motile sperms while percentage of viable sperm cells were significantly ($p < 0.05$) depleted at 540 µg/ml (pharmacologic concentration) at 4 hours (Figure 1). There was also a slight increase in the percentage of abnormal sperm cells at 0, 2 and 4 hours on exposure to ≥ 5.4 µg/ml CPFX.

The cytotoxicity of CPFX has been reported by some authors. This effect has been linked to its apoptotic-inducing potential and its ability to arrest cell cycle growth at the S phase. Changes in p53, Bcl-2 associated X protein (Bax) and B-cell lymphoma protein 2 (Bcl-2) expression in tested cell lines and in testicular tissues were reported following treatment with ciprofloxacin [16, 44, 46]. Growth inhibition of various cultured mammalian cells has also been reported with CPFX treatment. CPFX decreased cell viability in a dose- and time-dependent manner and at lower ciprofloxacin concentrations (0.01 mM and 0.1 mM) cells were arrested in S-phase suggesting a mechanism related to topoisomerase II inhibition. MDA levels were also increased in treated cell lines suggestive of an oxidative stress generation mechanism associated with its cytotoxic potential. [15, 16]. The cytotoxic and apoptotic effect of CPFX in rat testis has also been reported [45, 46].

We also report a significant ($p < 0.05$) dose and time-dependent increase in H_2O_2 and the lipid peroxidation product; MDA in boar semen exposed to CPFX, which must have elicited peroxidation of sperm membranes (Figure 2). This correlates with a significant decrease in SOD activity after 4hours of treatment with 54 µg/ml and 540 µg/ml of CPFX.

SOD belongs to the first line of endogenous antioxidant defences that are involved in the dismutation of superoxide anion (O_2^-) to H_2O_2 which is reduced to water by catalase and the GPX. Thus any biological system generating O_2^- will without question incur H_2O_2 as a result of a spontaneous dismutation reaction. In addition, some enzymatic reactions may produce H_2O_2 directly [47]. O_2^- itself can also react with H_2O_2 and generate the hydroxyl radical (OH^-). On another hand, in a succession of reactions called Haber-Weiss and Fenton reactions, H_2O_2 can break down to the hydroxyl radical (OH^-). OH^-, the most reactive of ROS, can damage proteins, lipids, carbohydrates and DNA. It can also initiate lipid peroxidation by the abstraction of an electron from polyunsaturated fatty acids [48]. The depletion of SOD activity observed in our study implies it is being overwhelmed by an over-increased production of O_2^- by CPFX and in its involvement in dismutating it to H_2O_2. On another hand, a decrease in sperm motility resulting from sperm mitochondria membrane damage and hence reduced adenosine triphosphate (ATP) production could be explained that the O_2^- anion and H_2O_2 generated by CPFX possibly resulted in OH^- the generation that led to lipid peroxidation of sperm membrane with resultant elevations in MDA levels. CPFX has been reported to generate radical species such as ROS and reactive nitrogen species (RNS) in testicular tissue and seminal fluid as a mechanism underlying its reproductive toxicity in males [4, 8, 22, 49, 50]. In the study of Xie *et al*. [22], CPFX induced oxidative damage evidenced by a significant elevation in MDA and a significant decrease in SOD content. Adedara *et al.* [4] also reports diminished endogenous antioxidant defences (SOD, CAT, GST and GPX activities as well as GSH, levels) with increased reactive oxygen and nitrogen (RONS) and LPO levels in the hypothalamus, testis and epididymis of CPFX treated rats. These studies and several others also report a reduction in sperm characteristics (motility, count and viability) as well as increased morphological abnormalities in the rats evidenced by the adverse impact of oxidative stress on the reproductive function of CPFX-treated rats [8, 9, 43].

Furthermore, in most of these studies, administration of CPFX adversely compromised markers of testicular tissue integrity, sex hormones and induced the up-regulation of some inflammatory biomarkers. The mid-piece and tail segments of spermatozoa of rats and elevated abnormalities in sperm morphology were also reportedly affected by CPFX treatment. Oxidative damage to the sperm tail and mid-piece (which houses the sperm structural machineries and the mitochondria; producing cellular energy for sperm motility and function) may consequently diminish the quality and life of sperm cells culminating in decreased fertilization of the ovum by the sperm [4, 9, 43].

Microsomal metabolism of CPFX generates free radicals [41] which may interfere with sperm mitochondrial ATP generation and membrane integrity decreasing motility and vitality [51]. Thus the decrease in sperm motility observed in our study could be purported to result from mitochondrial ATP depletion in sperm associated with mitochondria membrane damage and the reduced sperm cell viability as a result of CPFX-induced cytotoxicity of the spermatozoa population.

In our study, significant elevations were observed in GSH levels, CAT, GPX and GST activities in boar semen after incubation with CPFX for 4 hours. This observation was dose-dependent. Although most authors report diminished levels /activities of these antioxidants in a face of oxidative assault as in CPFX-induced tissue damage including testicular and sperm toxicity [4, 8, 17, 52], our results support similar observations in previous studies which report elevated antioxidant system activity in tissues (CAT in the liver and posterior kidney, GST in gills) of catfish *Rhamdia quelen* in short-term exposure to CPFX [53]. Also, CPFX alone and in combination with paracetamol, increased the levels of antioxidant enzymes and biomarkers of oxidative damage as well as altered the expression of Nuclear factor E2-related factor 1 and 2 (Nrf1 and 2), BAX, and Caspase 3, 6, 8, and 9 in the liver of the fish *Danio rerio* [54]. The result of our study indicates an adaptive response by the seminal antioxidant system in response to increased production of free radicals on exposure to CPFX [55].

In order to prevent oxidative/nitrosative stress, the cell must respond to ROS/RNS by mounting an antioxidant defence system. Therefore, redox regulation of transcription factors is significant in determining gene expression profile and cellular response to oxidative/nitrosative stress. Many hydrogen peroxide sensors and pathways are triggered by converging in the regulation of transcription factors including activator protein 1 (AP-1), Nrf2, antioxidant response elements (ARE), p53, Nuclear factor-kappa B (NF-κB) Notch (a membrane-bound transcription factor) e.t.c which induce the expression of a number of genes. Endogenous inducers including nitric oxide, hydrogen peroxide, lipid peroxidation products such as 4- hydroxyl nonenal (4HNE) and MDA, etc can react with specific cysteine residues in Keap1, leading to the release of Nrf2 and allowing its nuclear translocation, binding to the ARE and activating the expression of a battery of cytoprotective genes (antioxidant and detoxification genes). These ROS can also induce the transcription factors AP-1 and NF-kB with target genes mainly enzymes involved in the antioxidant response such as NAD(P) H:quinone oxidoreductase 1 (NQO1), glutathione S-transferases, ferritin heavy chain and SOD, etc [55]. Thus the increase in endogenous antioxidant activities (CAT, GPX and GST) and levels (GSH) observed in our study could probably be an initial adaptive response by the antioxidant defence system in an attempt to assuage CPFX-induced oxidative stress. This could possibly be by activation of transcription factors that trigger the expression of enzymatic detoxification and antioxidant genes and hence cellular enzymes as seen in previous studies with CPFX [53, 54].

4. Conclusion

CPFX has the potential to induce detrimental effects on sperm in a dose and time-dependent manner. This study shows that CPFX elicited increased ROS in semen which elevated intracellular antioxidant defenses as an initial adaptive response in an attempt to combat induced oxidative stress. Thus, it comes close that CPFX is able to induce its pathological effect even at physiological doses with prolonged usage.

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Abbreviations:

CPFX – Ciprofloxacin
CAS – Caspase
DNA – Deoxyribonucleic acid
AP-1 – Activator Protein 1
NQO1 – NAD(P)H:quinone oxidoreductase 1
MDA – Malondialdehyde
GSH – Reduced glutathione
SOD – Superoxide dismutase
O_2^- – Superoxide anion
OH^- – Hydroxyl radical
CAT – Catalase
H_2O_2 – Hydrogen Peroxide
GPX – Glutathione peroxidase
Nrf1– Nuclear factor E2-related factor 1
Nrf2 – Nuclear factor E2-related factor 2
ARE – Antioxidant response elements
NF-κB – Nuclear factor-kappa B
Bcl-2 – B-cell lymphoma protein 2
Bax – Bcl-2 Associated X protein
ROS – Reactive oxygen species
Keap1 Kelch-like ECH-associated protein 1
LH – Luteinizing hormone
FSH – Follicle stimulating hormone

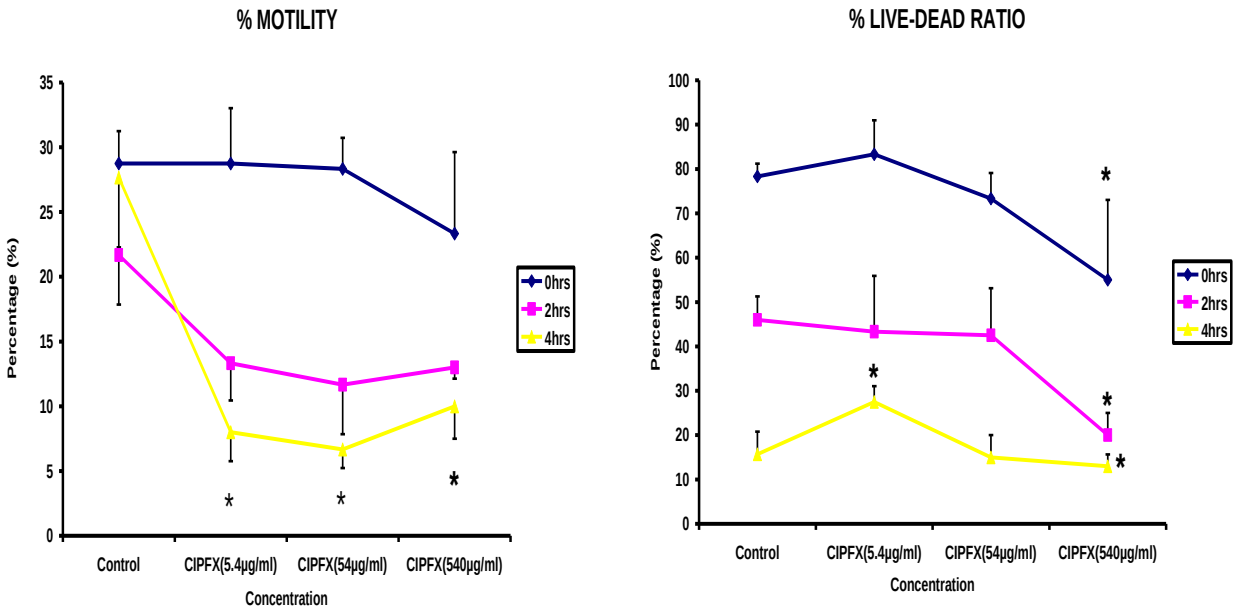
Table

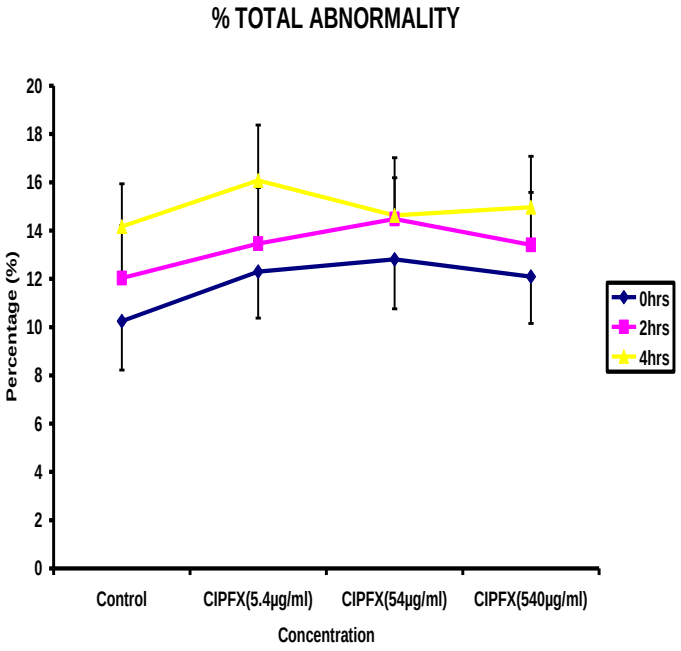
Table 2: Effect of CPFX Treatments on GSH, GST and GPX Levels in Boar Semen

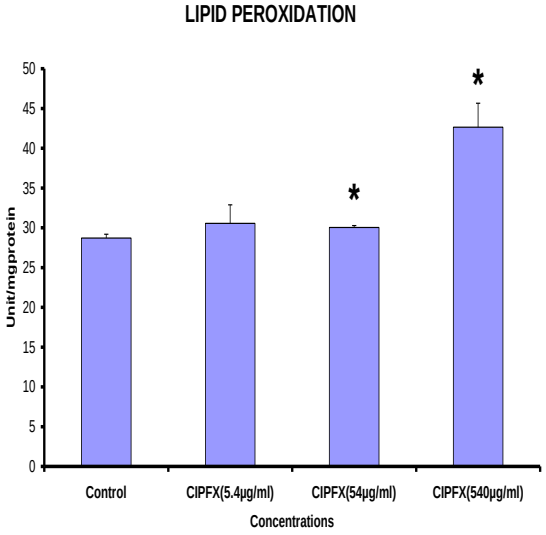
TREATMENTS	GSH (µg/mgprotein)	GST (µmol/min/mgprotein)	GPX (umol/min/mgprotein)
Control	5.90 ± 0.34	15.53 ± 0.39	12.98 ± 0.24
CPFX (5.4µg/ml)	6.50 ± 0.37*	21.79 ± 1.02*	13.30 ± 0.20
CPFX (54µg/ml)	6.86 ± 0.17*	27.77 ± 0.55*	13.52 ± 0.46*
CPFX (540µg/ml)	7.45 ± 1.74	35.31 ± 1.40*	16.89 ± 0.40*

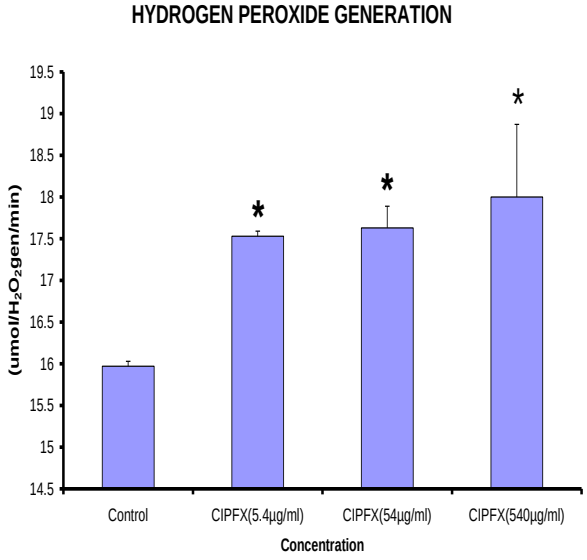
Values are mean ± SD, *P < 0.05 compared with control, n = 6, CPFX = Ciprofloxacin

Figures




% TOTAL ABNORMALITY
Percentage (%)
20
18
16
14
12
10
8
6
4
2
0
Control
ClPFX(5.4µg/ml)
ClPFX(54µg/ml)
ClPFX(540µg/ml)
Concentration
0hrs
2hrs
4hrs


LIPID PEROXIDATION
Unit/mgprotein
50
45
40
35
30
25
20
15
10
5
0
*
*
Control
ClPFX(5.4µg/ml)
ClPFX(54µg/ml)
ClPFX(540µg/ml)
Concentrations


HYDROGEN PEROXIDE GENERATION
(umol/H2O2gen/min)
19.5
19
18.5
18
17.5
17
16.5
16
15.5
15
14.5
*
*
*
Control
ClPFX(5.4µg/ml)
ClPFX(54µg/ml)
ClPFX(540µg/ml)
Concentration

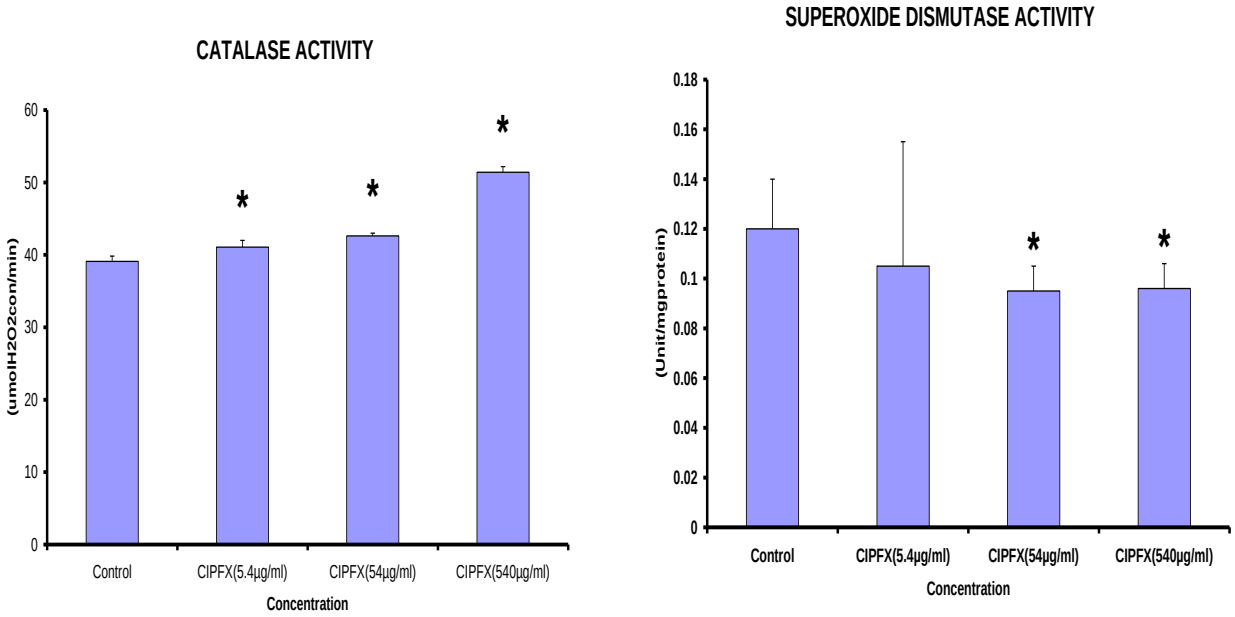


Figure Legends

Figure 1: Changes in sperm percentage (%) motility, live-dead ratio and total abnormality of boar semen at 0, 2 and 4hrs of incubation with various concentrations of CPFX. Values are mean ± SD, *P < 0.05 compared with control, n = 6, CPFX = Ciprofloxacin

Figure 2: Increased LPO, H_2O_2 levels and changes in the activities of CAT and SOD in boar semen after 4hrs incubation with various concentrations of CPFX. Values are mean ± SD, *P < 0.05 compared with control, n = 6, CPFX = Ciprofloxacin