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Adverse effects of ciprofloxacin on testis apoptosis and sperm parameters in rats

Arash Khaki[1] D.V.M., Ph.D., Mahnaz Heidari[2] M.Sc., Marefat Ghaffari Novin[3] M.D., Ph.D., Amir Afshin Khaki[4] Ph.D.

1 Department of Veterinary Pathology, Islamic Azad University of Tabriz, Tabriz, Iran.
2 Avesina Research Center, Tehran, Iran.
3 Cellular and Molecular Biology Research Center, Shaheed Beheshti University M.C., Tehran, Iran.
4 Department of Anatomical Science, Tabriz University of Medical Science, Tabriz, Iran.

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Abstract

Background: Ciprofloxacin is a commonly prescribed antibiotic in the treatment of genitourinary tract infection.

Objective: The aim of this study was to investigate the effects of ciprofloxacin on testis apoptosis and sperm parameters in rat.

Materials and Methods: Twenty male Wistar rats were selected and randomly divided into two groups; control (n=10) and experimental (n=10). The experimental group was orally received 12.5 mg/kg ciprofloxacin daily for 60 days and the control group just received water and food. Rats were then killed and sperm removed from cauda epididymis and analyzed for sperm motility, morphology, and viability. Testis tissues were also removed and prepared for TUNEL assay to detect apoptosis.

Results: Results showed that ciprofloxacin significantly decreased the sperm concentration, motility ($p<0.05$) and viability ($p<0.001$). In addition, ciprofloxacin treatment resulted in a significant decrease in the number of spermatogenic cells (spermatogonia, spermatocyte, spermatid and sperm) in the seminiferous tubules when compared with the control group. The apoptotic germ cells per seminiferous tubular cross section was significantly increased in the experimental group (15.11±3.523) as compared with the control group (7.3±0.762) ($p<0.05$).

Conclusion: It is concluded that ciprofloxacin has the toxicological effects on reproductive system in male rats.

Key words: *Ciprofloxacin, Testis, Sperm, Apoptosis, Rat.*

Introduction

In the last few years, a marked decrease in the quality of semen has been reported (1). These changes in semen quality are more likely to be due to environmental factors. Chemicals and drugs which are particularly misused are among these environmental factors (2). Antibiotics are commonly prescribed for a multitude of everyday condition. Not surprisingly, a proportion of male patients attending fertility clinics may have been prescribed antibiotics by their general practitioner to treat these unrelated infections (3). In addition, some patients requiring assisted conception occasionally show evidence of infection of the male reproductive tract (4, 5). The antibiotic ciprofloxacin is routinely used by urologists, andrologist and fertility specialists to treat such bacterial infections occurring prior to in vitro fertilization treatment, or when high concentration of leukocytes are present in the semen of these patients, irrespective of microbial evidence of infection (6).Ciprofloxacin is a synthetic antibacterial agent belonging to the family of fluoroquinolones with a very broad spectrum against of microbial pathogens, especially Gram-negative infectious diseases, that has been

Correspondence Author:
Marefat Ghaffari Novin, Cellular and Molecular Biology Research Center, Shaheed Beheshti University M.C., Tehran, Iran.
E-mail: mghaffarin@yahoo.com

approved in more than 100 countries world-wide (7). Ciprofloxacin is well absorbed orally and induced its antibacterial action mainly by inhibition of DNA gyras, which is equivalent to topoisomerase II in mammalian cell (8, 9). It is known that Ciprofloxacin can be transported to the seminal fluid and can directly affect sperm cells resulting in physiological, metabolic and /or genetic changes. Ciprofloxacin was detected in the prostatic tissue and seminal fluid in high concentration (10). In vivo genotoxicity studies suggest ciprofloxacin as safe for therapeutic use (6). However, other studies have demonstrated ciprofloxacin to significantly impair both testicular function and structure (11, 12). Administration of ciprofloxacin significantly reduced sperm count, motility and daily sperm production in rats (11). Demir *et al* also showed that in healthy rats, ciprofloxacin caused recognizable histological damage associated with a mild decrease in testicular volume and sperm concentration (12).

The aim of the present study was to define the effect of ciprofloxacin on sperm parameters and germ cells apoptosis in rat by using TUNEL assay for measurement of DNA fragmentation.

Materials and methods

Experimental animals

Twenty adult Wistar albino male rats 8 weeks old and weighing 250±10g were obtained from animal facility of Pasture Institute of Iran. All animals were treated in accordance to the Principles of Laboratory Animal Care (13). All rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to start of ciprofloxacin treatment in order to determine the amount of water needed per experimental animal.Thereafter, the animals were separated at random into two groups: experimental group received 12.5 mg/kg oral ciprofloxacin (Sigma 33433, USA) via gavage for 60 continuous days, the control group receiving only water and food. The dosages ciprofloxacin were similar to those used in human therapy. Body weight daily intake of food and water were determined several times per week throughout the study. All chemicals used in the present study were purchased from Sigma chemical.

Surgical procedure

On sixtieth day, the pentobarbital sodium (40 mg/kg) was administered intra peritoneal for anesthesia, and the peritoneal cavity was opened through a lower transverse abdominal incision. The reproductive organs (testes, epididymides and seminal vesicles) were carefully removed, washed in normal saline solution (0.9%), blotted and weighed. At the end of the experiment, the animals were anesthetized with diethyl ether and killed by decapitation according to recommendation of the Institutional Ethical Committee.

Epididymal spermatozoa motility, viability and count

Epididymal spermatozoa were collected by cutting the cauda region of the epididymis into small pieces in 5 ml of Ringer's medium at 37°C. A sperm viability test was done by the method described by World Health Organization (14). Assessment of sperm count and motility were performed according to Freund and Carol (15). Briefly, both cauda epididymis from each rat were placed in 2 ml of normal saline pre-warmed to 37°C. Small cuts were made in the two cauda epididymis where the spermatozoa were obtained and suspended in the saline solution. Two hundred microlitres of the suspension was diluted with 800 ml of saline. A small amount of the diluted suspension was transferred to both chambers of a Neubauer haemocytometer using a Pasteur pipette by touching the edge of the cover slip and allowing each chamber to be filled by capillary action.

Histopathology

Light microscopy

The testis was fixed in 10% formalin and embedded in paraffin. Five-micron thick sections were prepared and stained with hematoxylin and eosin (HE). The specimens were examined under Olympus/3H light microscope. Two perpendicular diameters of 10 seminiferous tubules were measured each in 10 slides (10×10=100 seminiferous tubules in each groups), at 40× magnification, with the aid of an ocular reticule standardized with a stage micrometer. Values were recorded as mean diameter of seminiferous tubule (16). The diameters of venous were also measured at the same way.

TUNEL analysis of apoptosis

The in-situ DNA fragmentation was visualized by TUNEL method (17). Briefly, dewaxed tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline solution (PBS) containing 3 % H_2O_2 for 10 min to block the endogenous peroxidase activity.

The sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP (in situ Cell

Death Detection, POD kit, Roche, Germany), for 60 min at 37°C. The slides were then rinsed three times with PBS and incubated with secondary antifluorescein-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine-H_2O_2 (DAB, Roche, Germany) chromogenic reaction was added on sections and counterstained with hematoxylin. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, and nucleotide mixture in reaction buffer was used instead. Apoptotic germ cells were quantified by counting the number of TUNEL stained nuclei per seminiferous tubular cross section. Cross sections of 100 tubules per specimen were assessed and the mean number of TUNEL positive germ cells per tubule cross- section was calculated.

Statistical analysis

Statistical comparisons were made using the Fisher -test for comparison of data in the control group with the experiment group. The results were expressed as mean ± S.E. (standard error). p-value less than 0.05 were considered significant.

Results

Weight of individual male reproductive organs

Weights of the testis, epididymis and seminal vesicle were significantly lower in treated animals with ciprofloxacin (12.5mg/kg/day), relative to the control group ($p<0.05$), which suggests that these antibiotics have the toxicity to male reproductive organs (Table I).

Table I. Male reproductive organ weights of control and treated rats.

Organs	Control (n=10)	Ciprofloxacin (n=10) (12.5 mg/kg)
Testis (gr)	1.53 ± 0.03	1.24 ± 0.03*
Epididymis (gr)	0.30 ± 0.025	0.20 ± 0.038*
Seminal vesicles (gr)	0.55 ± 0.016	0.19± 0.009*

Values are mean ± SE
*Significant different at $p< 0.05$ level, (compared with the control group).

Sperm count, viability and motility

Administration of ciprofloxacin 12.5mg/kg/day, orally, for 60 consecutive days significantly reduced sperm count and motility in experimental group as compared with control group ($p<0.05$) (Table II). Moreover, sperm viability was significantly declined in the treatment group when compared to the control group ($p<0.001$)(Table II).

Table II. The effect of ciprofloxacin on sperm count, motility and viability**.**

Groups	Total count (No. of sperm/rat×10^6)	Motility (%)	Viability (%)
Control (n=10)	60.8±0.20	48.4±2.03	85.1± 3
Ciprofloxacin (n=10)(12.5mg/k)	24.28±0.19*	34.2±0.8*	47.5±1.75***

Data are presented as mean±SE.
*Significant different at $p<0.05$ level, (compared with the control group).
***Significant different in viability at $p<0.001$, (compared with the control group)**.**

Table III. The number of spermatogenic cells in seminiferous tubules.

Cells	Control (n=10)	Ciprofloxacin (n=10) (12.5 mg/kg)
Spermatogonia	48.63±2.492	30.56±3.645*
Spermatocyte	67.50±4.222	50.87±4.654*
Spermatid	215.23±17.510	170.54±5.675*
Sperm	146.63±8.487	98.67±7.982*

Data are presented as mean±SD.
*Significant different at $p<0.05$ level, (compared with the control group).

Table IV. Diameter (µm) of the seminiferous tubule (ST) and vein in the control and treated animals.

Groups	Seminiferous tubule (ST)	Venus
Control	285±0.81	0.921±0.01
Experimental	250±0.77*	1.73 ±0.07*

Data are presented as mean±SE.
*Significant different at $p<0.05$ level, (compared with the control group).

Light microscopic study

Histopathological study showed the cycle of spermatogenesis was regular in all males in the control group (Figure A). However, ciprofloxacin treatment resulted in a significant decrease in the number of spermatogenic cells (spermatogonia, spermatocyte, spermatid and sperm) in the seminiferous tubules (Figure B) (Table III). Intertubular spaces and veins congestion were increased in the treatment group as compared with those seen in the control group. Morphometeric study showed the diameter of seminiferous tubule (ST) was (250±0.77) and (285±0.81) µm in the experimental and control group, respectively.

This data is shown the diameter of (ST) was decreased in the experimental group when compared with the control group (Table IV). The diameter of veins was 0.921±0.01 µm and 1.73 ±.07 µm in the control and the experimental groups, respectively.

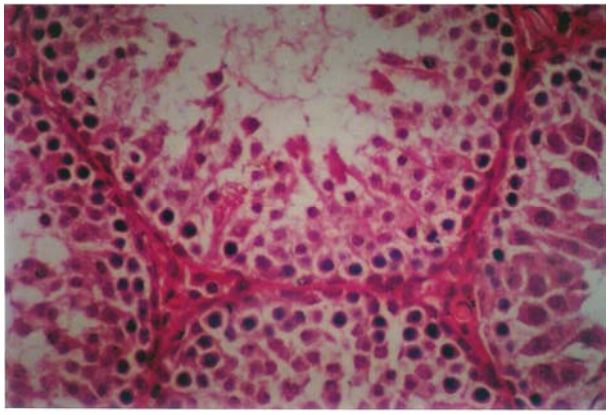

Figure A. Testicular section from a control rat shows the normal spermatogenesis and histological structure of the seminiferous tubules and intertubular spaces (H and E 40 ×).

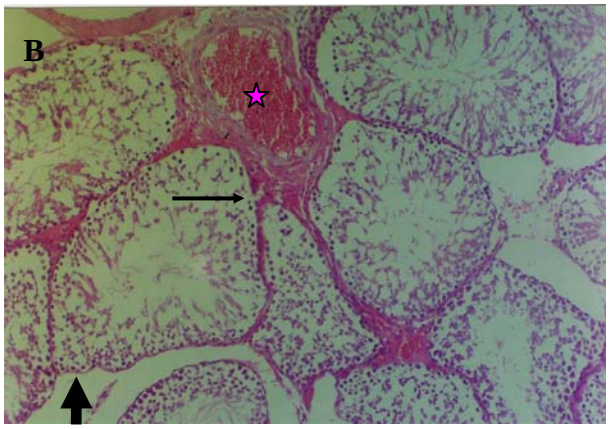

Figure B. Testicular section from a treated rat with ciprofloxacin (12.5 mg/kg) shows a decrease in the number of spermatogenic cells in the seminiferous tubules with intertubular spaces (triangle) and veins (star) congestion (H and E 40 ×).

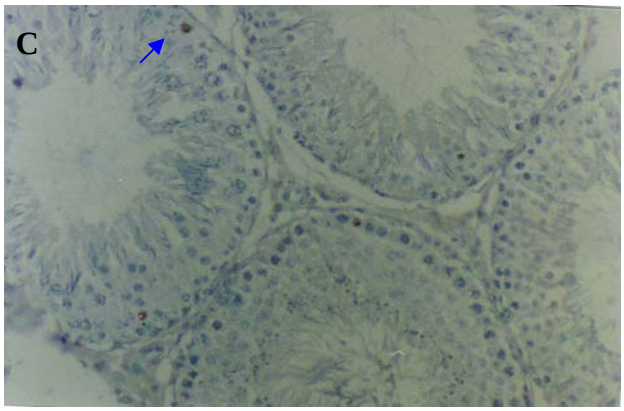

Figure C. Testicular section from a control rat shows apoptosis in primary spermatocyte that dark brown apoptotic body (arrow) detecting by TUNEL assay, (40×).

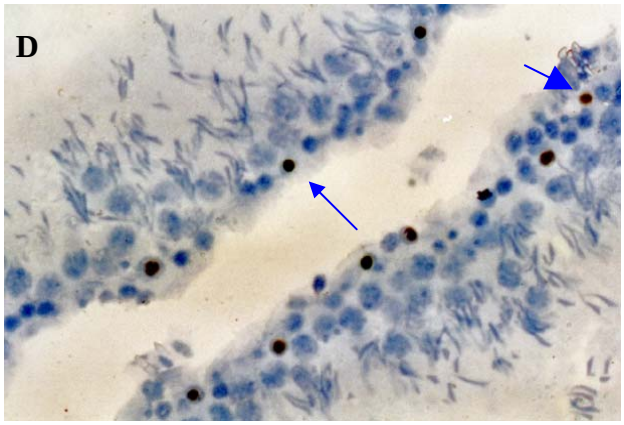

Figure D. Testicular section from a treated rat with ciprofloxacin (12.5 mg/kg) shows apoptosis in primary spermatocyte (arrow) and spermatogonia cells (triangle) that detecting by TUNEL assay, (40×).

Result of apoptotic germ cells

In this study, the number of TUNEL positive germ cells per tubule cross-section increased following ciprofloxacin treatment compared to the control group. The TUNEL positive spermatogonia and spermatocytes were the main germ cells undergoing apoptosis. Figures C and D demonstrate the typical histograms of TUNEL assay analyzed by light microscope in both groups. Using the in situ detection method by which the apoptotic cells can be identified by their darkly stained nuclei, we observed a low incidence of spontaneous apoptosis in normal rat testis from the control group. Although in low numbers, apoptotic germ cells were also observed in the control rats. The rate of total apoptotic cells (spermatogonia and spermatocytes) were 15.11±3.523 and 7.3±0.762 in the experimental and the control groups, respectively according to the definition of Clermont method (18).

Discussion

The therapeutic and prophylactic effects of ciprofloxacin on several infections have been well documented. However, the results of our experimental study reveal that prolonged administration of therapeutic dose of ciprofloxacin was promoted male reproductive toxicity in rats. Degenerative changes in the seminiferous tubules and reduction in sperm count and motility are the evidence for this toxicity.

The present study indicated that, administration of ciprofloxacin for 60 consecutive days, results in a marked reduction in sperm count, sperm motility and viability as compared to respective controls. This is in agreement with that of Abd-Allah *et al*, who reported that ciprofloxacin treatment for 15 days in rats caused in a marked reduction in sperm count and motility (11). In addition, Demir *et al*, has similarly shown that ciprofloxacin treatment for 10 days in rats resulted in a marked

reduction in sperm count and motility (12). Previous studies in which male patients were given 250 mg ciprofloxacin twice a day did not show differences in sperm quality (19) and was without effect of spermatogenesis (20). In contrast, at pharmacologic concentration (100× physiologic), ciprofloxacin adversely affected human sperm motility with decrease rapid progression in vitro (21). The reasons for the discrepancy may be due to differences between in vitro and in vivo studies. Thus, up to 20% of each ciprofloxacin dose is metabolized in vivo, and ciprofloxacin metabolites may exert some biological activities that are not detectable by in vitro assays (22). On the other hand, the possible release of bacterial factors, from indigenous bacteria killed by the drug, should not be neglected.

It has been reported that the decrease in sperm count and motility are valid indices of male infertility in laboratory animals (23, 24). However, sperm motility is often used as a marker of chemical-induced testicular toxicity (25). They have also stated that the disruption of seminiferous epithelium is indicative of male reproductive hazard. Therefore, our experimental results suggest a gonadotoxic potential of ciprofloxacin. One of the reason for this effect can be explained on the basis that, ciprofloxacin interferes with the energy production process required for sperm vitality and motility (26). Apoptosis of select germ cells occurs normally in the testis and is essential for the normal maintenance of spermatogenesis (27-29). However, the relatively small increase in the percentage of germ cell apoptosis can result in defective spermatogenesis leading to infertility (30). Increases in the incidence of germ cell apoptosis often are observed as a result of various forms of physical or chemical injury to the testis (31). The present study is demonstrated that apoptosis in male rat germ cells is induced by ciprofloxacin. These results indicate that ciprofloxacin like other chemical agents may directly interfere in the process of spermatogenesis. This increase in germ cell apoptosis is possibly partly due to an increased peroxide radical generation in the testis following ciprofloxacin treatment (32). This increase then induces DNA single-strand breaks and chromosomal aberrations as demonstrated by in vitro genotoxicity studies (33, 34). In addition, previous study showed that caspase-3, which has an important role in apoptosis, could be activated by ciprofloxacin (35, 36). Therefore, ciprofloxacin could also induce apoptotic pathways through activation of Caspases. In conclusion, this study may suggest that ciprofloxacin has potential testicular toxicity as evidenced by decreased sperm count and motility, increase apoptotic cells and pathological testis changes.

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