Reproductive Sciences
20(9) 1090-1095

DOI: 10.1177/1933719113477482
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Role of Mitochondria in Ciprofloxacin-Induced Apoptosis in Murine Sperm Cells

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Abstract

Rationale: Ciprofloxacin (CPFX) has been reported to inhibit cell growth and induce apoptosis in certain eukaryotic cells. The role of the mitochondrial pathway in CPFX-induced apoptosis in cultured murine sperm cells was investigated. **Methods and Results:** Sperm cells (5×10^3 cells/well) from 8-week-old NMRI male mice were cultured in 150 μL of HAM's F10 with 25 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES and 10% human serum albumin and were incubated with 50, 100, 200, 400, and 800 μg/mL CPFX for 24 and 36 hours. Cell cytotoxicity, mitochondrial membrane potential ($\Delta\Psi M$), and concentrations of caspase 3 and caspase 9 were assessed in CPFX-treated cultured murine sperm cells by MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide) , JC-1 (5, 5á, 6, 6á-tetrachloro-1, 1á, 3, 3á-tetraethylbenzimidazol-carbocyanine iodide)aggregation, and caspase 3 and caspase 9 assays, respectively. Increasing doses of CPFX showed significant cytotoxicity ($EC_{50} = 146.73$ μg/mL). Significant loss of $\Delta\Psi m$ was observed in sperm cells treated with $\geq$50 μg/mL CPFX for 36 hours. Significant increases in caspase 9 and caspase 3 concentrations were also found in cells treated with $\geq$50 μg/mL CPFX for 24 and 36 hours, respectively ($P < .001$). **Conclusions:** Effects of clinically reachable doses of CPFX on cultured murine sperm cells were investigated and revealed that it may cause sperm cell toxicity by induction of apoptosis through the mitochondrial pathway in the clinically reachable concentrations.

Keywords

ciprofloxacin, apoptosis, mitochondrial membrane potential, caspases, sperm cells

Introduction

Ciprofloxacin (CPFX) is a second-generation fluoroquinolone broad-spectrum antibiotic used to treat a number of gram-positive and gram-negative bacteria, such as infections of bones and joints, respiratory tract infections, and urinary tract infections.[1,2] It acts mainly by inhibiting a type II topoisomerase, DNA gyrase, which is necessary for the unwinding of replicated prokaryotic DNA.[2] It has been shown, however, that this mechanism can also affect mammalian cell replication through the inhibition of eukaryotic topoisomerases.[3] Although quinolones are highly toxic to mammalian cells in culture, their mechanism of cytotoxic action is not fully understood, and it continues to be debated whether such effect is to be considered one of the mechanisms of severe adverse reactions experienced by some patients following CPFX therapy.[4] Ciprofloxacin has been reported to significantly impair both testicular function and structure in rats.[5,6]

On the other hand, prospects of new applications for CPFX in the treatment of malignancies have been reported in several in vitro studies on human bladder cells,[7,8] leukemic cell lines,[9] human osteosarcoma cells,[10] human prostate cancer cells,[11] human colorectal carcinoma cells,[12] and a human non–small-cell lung cancer cell line.[13] The molecular mechanism of the anticancer action of CPFX is not yet fully understood, but several studies have shown that CPFX may target nuclear topoisomerase II.[14] However, the cell-killing behavior of CPFX is different from those of nuclear topoisomerase II-acting drugs. A high concentration of CPFX is needed to inhibit the activity of topoisomerase II, and only cell proliferation arrest has been observed in the presence of low CPFX concentrations.[15] Hence, it seems that additional mechanisms are responsible for CPFX cytotoxicity, which can be connected to its action at the levels of cell membrane and mitochondria.

The CPFX has the ability to inhibit cell proliferation through mitochondrial DNA damage. The CPFX has also been reported

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to react with the mitochondrial topoisomerase II isoform and then inhibit mtDNA synthesis, which finally leads to mitochondria damage and the depletion of intracellular adenosine triphosphate (ATP) resources with subsequent apoptosis.[16,17] The effect of CPFX (206 mg kg^{-1}, PO for 45 consecutive days) on the quality of murine sperm cells and early embryonic development was recently published by the authors, which showed that it induces DNA damage and chromatin abnormalities in the sperm cells, which consecutively leads to the low fertilization rate and retarded embryonic development.[18] This in vivo effect of CPFX could be secondary to the effect of CPFX on the male gonadal tissue, but the direct effect of CPFX on sperm cells is another alternative, which the current study sought to evaluate through the assessment of the CPFX-induced mitochondrion-dependent apoptotic pathway in cultured murine sperm cells.

Methods

Isolation and Culture of Sperm Cells

All procedures utilizing mice were performed according to the specific rules provided by the Animal Care and Use Committee of Veterinary Collage of Urmia University. Three mature healthy 8-week-old male NMRI (Naval Medical Research Institute) mice were sacrificed, and both epididymis of each male were transferred to a 60-mm petri dish containing 1 mL of prewarmed HAM's F10 with 25 mmol/L HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 10% Human Serum Albumin (HAS). The Cauda was minced, making 5 to 7 slashes with a 30-gauge needle of an insulin syringe. After incubation for 30 minutes at 37°C with 5% CO_2, the ground epididymal tissue was separated from the released spermatozoa. The purity of the sperms was checked by microscopic observation of Giemsa stained slides by 3 independent observers and the number of sperm cells was counted using hemacytometer, then 5×10^3 cells were dispensed into each well of 96-well microtiter plates containing HAM's F10 with 25 mmol/L HEPES and 10% HSA, and incubated with 50, 100, 200, and 400 μg/mL CPFX (17850, >98%, Sigma-Aldrich, Chemie Gmbh, Munich, Germany). The cultures were maintained at 37°C in a 5% CO_2-humidified atmosphere. Sperm cells collected from each mouse were treated and evaluated separately, and all markers were assessed after 24 and 36 hours of incubation for at least 3 times.

Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of CPFX in cultured sperm cells by calculating the half-maximal effective concentration (EC50) value. It is a colorimetric assay for measuring the activity of mitochondrial enzymes that reduce MTT to formazan dyes, giving a purple color, but it mainly allows assessing the cytotoxicity of potential agents that are capable of stimulating or inhibiting cell viability and growth.

Spermatozoa were dispensed into 96-well microtiter plates (5×10^3 cells/well) containing 150 μL of HAM's F10 with 25 mmol/L HEPES and 10% HSA and were incubated with 50, 100, 200, 400, and 800 μg/mL CPFX for 24 hours. Untreated wells were considered as control cells. The assays were carried out as described previously,[19] but dimethyl sulfoxide (DMSO) was used instead of acid-isopropanol. Briefly, cell viability was assessed by incubating cells with 0.5 mg/mL of MTT for 4 hours. The MTT formazan produced by viable cells was then dissolved in DMSO. The optical density values were read at 540 nm using Microplate Reader (ELx 800, BioTek Germany, Bad Friedrichshall, Germany). The optical density values were presented as the relative numbers of viable cells. All experiments were repeated at least 3 times.

Analysis of Mitochondrial Membrane Potential

Mitochondrial injury was assessed using the JC-1 Mitochondrial Membrane Potential Assay kit (Cayman Europe, Tallinn, Estonia). In healthy cells with high mitochondrial membrane potential ($\Delta\Psi m$), JC-1 (5, 5á, 6, 6á-tetrachloro-1, 1á, 3, 3á-tetraethyl benzimidazol-carbocyanine iodide) spontaneously forms complexes known as J-aggregates with intense red fluorescence at 595 nm. However, in apoptotic or unhealthy cells with low $\Delta\Psi m$, JC-1 remains in the monomeric form with green fluorescence at 535 nm. So, high $\Delta\Psi m$ (live) cells /low $\Delta\Psi m$ (apoptotic) cells ratio could be estimated from the ratio of florescence intensity of J-aggregate at 595 (red)/florescence intensity of monomeric JC-1 at 535 (green). The JC-1 staining solution was prepared by diluting the reagent at a 1:10 ratio in the culture medium. The sperm cells (5×10^3 cells/well) were treated with 10 μL of the JC-1 staining solution after 24 and 36 hours of incubation with different concentration of CPFX between 50 and 400 μg/mL. The samples were then incubated in a CO_2 incubator at 37°C for 30 minutes. The precipitated cells (400 g) were resuspended in assay buffer, and the fluorescence intensity of the samples was measured by spectrofluorometer (Hitachi F-2500 Hitachi High Technologies America, Inc, Dallas, Texas) at 595 nm for J-aggregates in healthy cells and at 535 nm for apoptotic cells. All experiments were repeated at least 3 times and fluorescence intensity of the reagents without sperm cells were considered as negative control. The results were expressed using the following equation:

$$\frac{\text{Fluorescence intensity at 595 nm}}{\text{Fluorescence intensity at 535 nm}} \approx \frac{\text{High } \Delta\ \Psi\ \text{m (Live) cells}}{\text{Low } \Delta\ \Psi\ \text{m (apoptotic) cells}}$$

Assessment of Caspase 3 and Caspase 9

Concentrations of caspase 3 and caspase 9 were measured in CPFX-treated and control sperm cells using mouse enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science Inc, China). In these kits, the microtiter plate provided is precoated with an antibody specific to caspase 3 or caspase 9. Standards or samples were later added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal

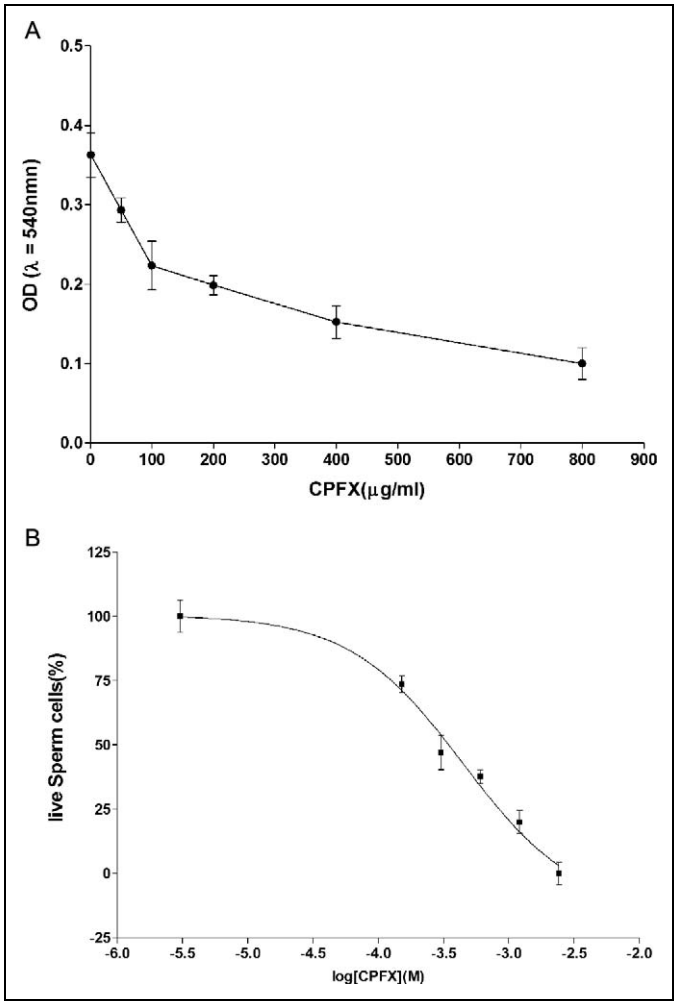

Figure 1. The cytotoxicity of ciprofloxacin (CPFX) in sperm cells collected from 3 treated mice with different concentrations for 24 hours using MTT assay. A, Ciprofloxacin decreased the number of cells in a dose-dependent manner. Data represent the average values (mean ± standard deviation [SD]) from 3 independent experiments. B, The dose-response curve was constructed using GraphPad Prism version 3.02. The EC_{50} is 146.73 μg/mL.

antibody preparation specific for caspase 3 or caspase 9 and then avidin-conjugated horseradish peroxidase was added and incubated. Subsequently, a 3,3′,5,5′-tetramethylbenzidine substrate solution was added to each well. Change in color was observed only in those wells that contained caspase 3 or caspase 9, biotin-conjugated antibody, and enzyme-conjugated avidin. Sulfuric acid solution was used as stop solution to terminate the enzyme–substrate reaction, and the color change was measured at 450 nm using a microtiter plate reader (Biotek ELX 808, BioTek Germany, Bad Friedrichshall, Germany). The concentration of caspase-3 or caspase-9 in the samples was then determined by comparing the optical density of the samples with the corresponding standard curve. The minimum detectable dose of mouse Caspase 3 and Caspase 9 was less than 0.058ng/mL and 0.061ng/mL, respectively. Intra-assay and Interassay coefficient of variance for both kits were less than 12%. The negative and positive controls were the wells without cell homogenate and premixed homogenate with defined amount of caspase 3 or 9 proteins, respectively. The results were normalized by dividing caspase concentrations by the protein content of each sample measured by method of Bradford.[20] All experiments were repeated at least 3 times they were expressed as ng Caspase 3 or 9 per mg protein ± standard deviation (SD).

Statistical Analyses

All experiments were repeated at least 3 times and the results are represented as mean, ratio or percentage ± SD. Dose–response and other curves as well as statistical significance were determined by analysis of variance (ANOVA) and subsequently applying the Tukeys multiple comparison test using GraphPad Prism (version 3.02 for Windows, GraphPad Software, San Diego, California) and P less than .05 was considered significant.

Results

Cytotoxicity Assay

The cell toxicity of CPFX between 50 and 800 μg/mL was investigated in sperm cells treated for 24 hours. CPFX elicited a significant cytotoxicity on cultured sperm cells in a dose-dependent manner (Figure 1A), with an EC_{50} of 146.73 μg/mL (Figure 1B).

The calculated EC_{50} value was used to select the range of CPFX doses for the other experiments.

Mitochondrial Membrane Potential Assay

The ΔΨm of cells treated with different doses of CPFX for 24 and 36 hours was significantly lower than that of controls ($P <$ .01 and $P <$ .001, respectively; Figure 2). The loss of ΔΨm in sperm cells treated with ≥50 μg/mL CPFX was significantly different from that of control cells after 36 hours, but a significant loss of ΔΨm was observed after 24 hours in the cells receiving ≥100 mg/mL CPFX.

Caspase 9 Assay

The concentration of caspase 9 in sperm cells treated with different doses of CPFX between 50 to 400 μg/ml for 24 or 36 hours was significantly higher than that of controls (ANOVA, $P <$ .001; Figure 3A). The increase in caspase 9 concentration in cells treated with ≥50 μg/mL CPFX for 24 hours was significantly higher than that in control cells ($P <$ .001). A significant correlation was observed between dose and response for doses of 50 to 200 μg/mL ($P <$ .001), but it was not significant for doses >200 μg/ml ($P >$ .05). The EC_{50} of CPFX for induction of caspase-9 was 123.86 μg/mL. Caspase 9 concentration in cells treated for 36 hours was also significantly higher than that in controls ($P <$ .001), but no significant differences were found between doses >50 μg/mL ($P >$ .05). Furthermore, treatment of cells for 24 hours seemed to be enough for induction of caspase 9, and it showed better discrimination among doses than did treatment for 36 hours.

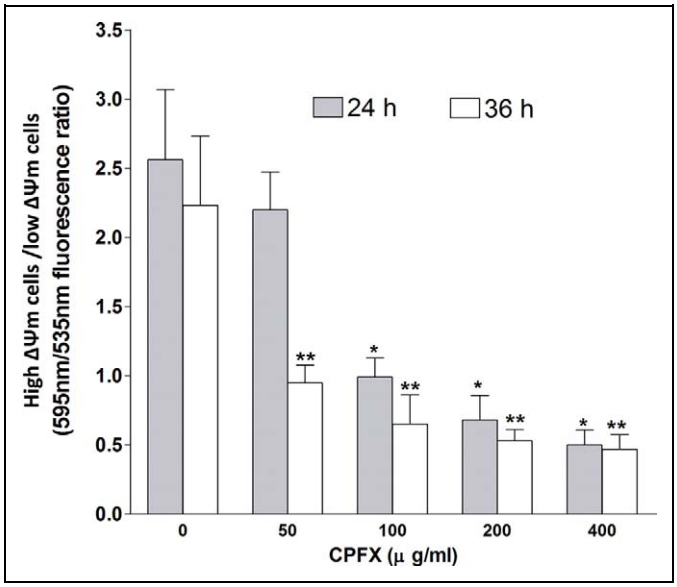

Figure 2. Effects of ciprofloxacin (CPFX) on mitochondrial transmembrane potential ($\Delta\Psi$m) in cultured murine sperm cells collected from 3 mice. After treatment with different concentrations (50-400 μg/mL) of CPFX for 24 and 36 hours, fluorescence intensity at 595 nm (red) and at 535 nm (green) was measured directly and red/green fluorescence ratio was assigned as high $\Delta\Psi$m cells /low $\Delta\Psi$m cells ratio. Data represent the average values (mean ± standard deviation [SD]) from 3 independent experiments. Data were analyzed using analysis of variance (ANOVA).* $P < .01$ CPFX (≥100μg/mL for 24 hours) versus controls, ** $P < .001$ CPFX (≥50μg/mL for 36 hours) versus controls.

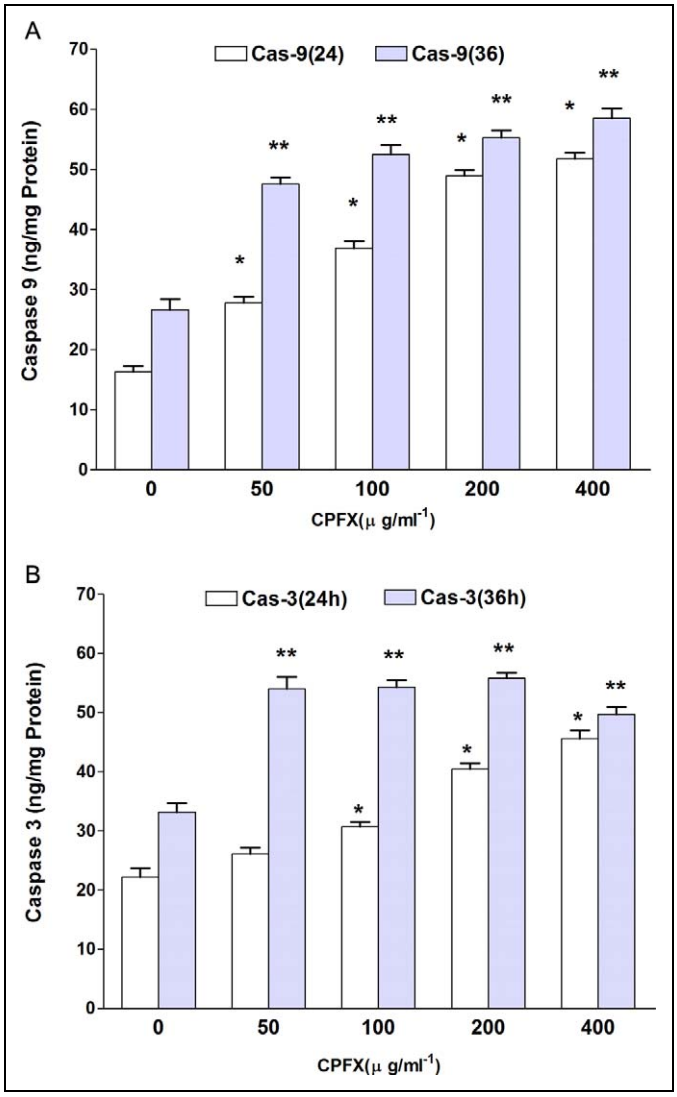

Figure 3. Concentration of caspase 9 and caspase 3 in cultured murine sperm cells collected from three mice was quantified following treatment with various concentrations of CPFX (50-400 μg/ml) by an enzyme-linked immunosrbent assay (ELISA) and was normalized using protein content of each sample. Data represent the average values (mean ± standard deviation [SD]) from 3 independent experiments. Data were analyzed using analysis of variance (ANOVA). A, The concentration of caspase 9 increased in concentration-dependent manner,* $P < .001$ CPFX (≥50 μg/mL for 24 hours) versus controls. ** $P < .001$ CPFX (≥50μg/mL for 36 hours) versus controls. $EC_{50} = 123.86$ μg/mL. B, Caspase 3 increased in a concentration-dependent manner, * $P < .01$ CPFX (≥100 μg/mL for 24 hours) versus controls. ** $P < .001$ CPFX (≥50 μg/mL for 36 hours) versus controls $EC_{50} = 198.63$ μg/mL.

Caspase 3 Assay

The concentration of caspase 3 in sperm cells treated with different doses of CPFX between 50 and 400 μg/mL for 24 hours was significantly higher than that in controls (ANOVA, $P < .001$; Figure 3B). The increase in caspase 3 concentration in cells treated with ≥100 μg/mL CPFX for 24 hours was significantly higher than that in control cells ($P < .01$). The EC_{50} of CPFX for induction of caspase 3 was 198.63 μg/mL. The increase in caspase-3 concentration in cells treated with ≥50 μg/mL CPFX for 36 hours was significantly higher than that in controls ($P < .001$), suggesting that 36 hours of treatment of the cells with dose of CPFX more than 50 μg/mL is required for induction of caspase 3.

Discussion

Drug-induced infertility and sexual dysfunction have been reported for several therapeutic, hormonal, or toxic agents.[21-23] Regarding CPFX-induced infertility, it has been issued by the manufacturer who performed fertility studies in rats at oral doses of CPFX up to 100 mg/kg (approximately 0.7-times the highest recommended therapeutic dose based upon mg/m^2) revealed no evidence of impairment.

On the other hand, CPFX has been reported to significantly impair both testicular function and structure in rats.[5,6] Mice treated daily with different doses of CPFX have been found to have significantly immature sperm cells, with a higher amount of single-stranded DNA.[18]

In the present study, we found that treatment of cultured murine sperm cells with CPFX induces apoptotic cell death in a dose- and time-dependent manner.

Apoptosis not only has critical role in the development of testicular tissue,[24-28] but also occurs in the mature sperm cells

in response to different kinds of apoptosis-inducing factors, and it has been proved that the mature human and animal sperm cells possess functional components of apoptotic machinery[29-31] instead of the general belief that sperm cells have a modified type of protein synthesis.[32-34]

It has been documented that during apoptosis, several key events occur in mitochondria, including the release of caspase activators such as cytochrome c, changes in electron transport, and loss of $\Delta\Psi m$.[35] For this reason, $\Delta\Psi m$ is an important parameter of mitochondrial function and has been used as an indicator of cell health. The data presented in this study demonstrate that CPFX causes a significant reduction in $\Delta\Psi m$ of sperm cells which is reflected by the reduction in J aggregate formation and related red fluorescence intensity in JC-1 mitochondrial membrane potential assay. Mitochondrial potential changes induced by CPFX in certain eukaryotic cells have been previously described.[12]

Fluoroquinolone antibiotics may interfere directly with mitochondrial membrane proteins or trigger the Bax pathway of apoptosis.[12,36,37] Aranha et al showed an upregulation of Bax in a CPFX-treated bladder carcinoma cell line; this altered the Bax-Bcl-2 ratio, which may be responsible for activation of the mitochondrial permeability transition pore with subsequent mitochondrial depolarization.[17]

Furthermore, our study indicates that a decline in $\Delta\Psi m$ was accompanied by caspase 9 and caspase 3 activation in a dose- and time-dependent manner. One of the major components of the apoptotic pathway involves the activation of caspase, a class of aspartic acid–directed cysteine proteases. Caspase 9 appears to be the initiator of caspase activation in the mitochondrial apoptotic pathway. As reported previously, CPFX treatment results in the release of cytochrome c as a consequence of mitochondrial potential changes; cytochrome c, in turn, interacts with Apaf-1, dATP/ATP, and procaspase 9 to form a complex known as the apoptosome.[17] Once activated, caspase 9 can activate the downstream executioner caspase, including caspase-3, leading to apoptotic cell death.[12,17] the role of caspase 9 and caspase 3 in the apoptosis of testicular germ cells has been investigated in different in vivo and in vitro studies.[38,39]

The currently approved dosage for intravenous CPFX in the United States is 200 to 400 mg given every 12 hours. Recently a higher dosage regimen of 400 mg every 8 hours for the treatment of severe respiratory tract infections was investigated and showed that the plasma level of CPFX could be 6.83 ± 17 mg/L.[40] The observed steady-state maximum CPFX plasma concentrations in the patients treated with high-dose CPFX (800 mg) intravenously every 12 hours, on treatment day 8 were 13 μg/mL. Regarding blood–testis barrier, it has been reported repeatedly that CPFX levels in semen are higher than of the serum concentrations and could be 7 times higher than plasma.[41-43] By taking the mentioned points into account, it seems possible to reach more than 50 mg/mL in semen of the patients receiving high-dose of CPFX.

Although, significantly negative impact of CPFX in the fertilization rate of sperm cells obtained from the CPFX treated murine testicular tissues has been shown in the in vivo study.[18] Regarding the reversibility of the toxic effect, this is an in vitro study that just shows direct impact of CPFX on the sperm cells and that the events like induction of apoptosis are irreversible. However, most of the drug-related side effects, with the exception of genotoxicity and teratogeny, have transient instead of reversible nature and the effects are mainly limited to the period of time during which the concentration of a drugs or chemicals is enough to cause side effects. Hence, the observed effect of the ciprofloxacin on the sperm cells could be considered as transient but not a reversible effect.

In summary, the findings of the current study provide new evidence of the ability of CPFX to induce apoptosis in sperm cells in the clinically reachable concentrations, which is mediated through the induction of mitochondrial potential changes and activation of caspase 3 and caspase 9. This is an experimental study using animal model and further studies are required to show the effect of CPFX and EC50 value for human sperm cells. Epidemiological studies are also needed to evaluate the prevalence of CPFX-related male genital dysfunction and its impact on the public burden of male infertility. The effect of CPFX on the expression of apoptosis-related genes remains for further investigations as well.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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