Full Length Research Paper

Effect of a calcineurin inhibitor tacrolimus (FK506) treatment on meiotic chromosomes in testes, epididymal spermatozoa and fertility in Swiss Albino male mice

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Tacrolimus hydrate (FK506), isolated from *Streptomyces tsukubaensis*, is an immensely used immunosuppressive agent. It was evaluated for its effects on meiotic chromosomes in testes, epididymal spermatozoa and fertility in male mice. The study under different parameters constituted sub acute (seven days) administration (gavage) of FK506 at doses of 4, 8 and 16 mg/kg/day body weight. The results obtained in present study revealed that, FK506 significantly induced spermatozoa abnormalities, lowered fertility and increased embryonic loss. The observed changes related to spermatogenic dysfunction reflected statistically significant increased aberrations in the meiotic chromosomes. These aberrations seemed to be induced by the inhibitory effect of the drug on the signal transduction pathways in the cells and interference in the transcription processes and proliferation of cells. Further studies are warranted to evaluate the safe dose and duration of therapy to determine the exact mode of action of FK506 induced germ cell toxicity.

**Key words:** Tacrolimus (FK506), sperm quality, germ cell toxicity, embryonic loss.

INTRODUCTION

Tacrolimus hydrate (FK506 or Fujimycin) (Figure 1) is a naturally occurring macrolide antibiotic, isolated from a soil fungus *Streptomyces tsukubaensis*. It was found to be a potent immunosuppressive drug (Hopkins and McNeil 2008; Gensburger et al., 2010). The use of cyclosporine A and FK506, both calcineurin inhibitors, led to major advances in the field of transplantation, with excellent short-term outcome (Naesens et al., 2009). FK506 was found to have similar mechanism of action as that of cyclosporine. However, cyclosporine treatment significantly reduced the number of metabolically active osteoblast-like cells, which were associated with excessive bone loss when compared with FK506 treatment

(Yoshikawa et al., 2005; Moreira et al., 2009). It is used worldwide primarily for the prophylaxis of liver, heart and kidney allograft rejection (Morales et al., 2005; Webster et al., 2005; Flechner et al., 2008; Sanchez-Lazaro et al., 2010; Vermeulen et al., 2010). FK506 was found to be both effective and safe in treating active rheumatoid arthritis patients with complicated backgrounds in clinical practice (Schwartz et al., 2006; Suzuki et al., 2009).

Besides successful use of FK506 in heart, liver, kidney and pancreas transplant (Neal et al., 2001; Flechner et al., 2008; Naesens et al., 2009; Girman et al., 2010), it was also incorporated in more potent immunosuppressive regimens needed in lung transplant cases (Harrison et al., 2007; Snell and Westall, 2007). In children with liver transplant, FK506 treatment did not disturb lipid metabolism and body antioxidant status (Wierzbicka et al., 2007). It protected neuronal tissue from hypoxic insults (Noto et al., 2007). The neuroprotective activity of

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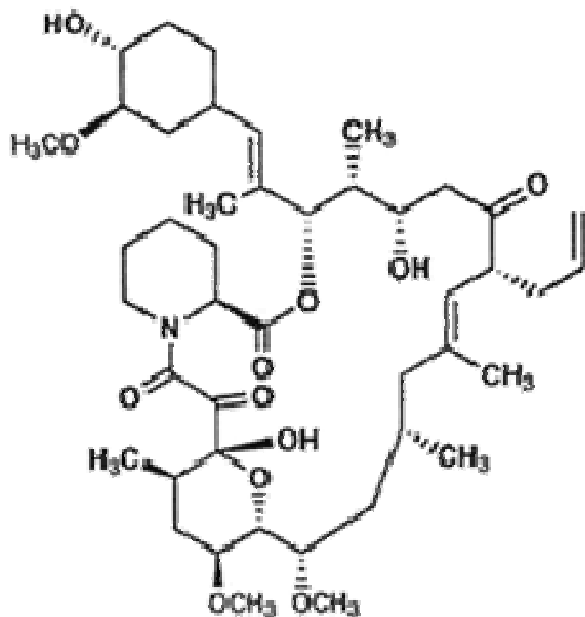
Molecular formula; C44H69NO12

Figure 1. Structure of tacrolimus (FK506).

FK506 was associated to its inhi-bitory effect on glutamatergic neurotransmission (Szabo et al., 2010). It markedly reduced the activity and number of liver-resident natural killer cells and supported liver regene-ration. In cases of heart transplantation, FK506 exhibited no disturbance in interstitial pressure or microcirculation, which might occur due to oedema of the grafted organ (Johnsson et al., 2004). FK506 also showed a tendency for longer time to first rejection and allowed fewer viral infections. In addition, patients developed less hyper-tension and needed lesser drugs for its control (Sanchez-Lazaro et al., 2010). After kidney transplantation, FK506 improved graft survival; however, it caused post-transplant diabetes, neurological, nephrotoxic and gastro-intestinal side effects and hypomagnesaemia (Ferraris et al., 2004; Kim et al., 2006; Kovarik and Slade 2010; Wu et al., 2010).

A combined preparation of FK506 derivatives and beta.2-agonist prevented acute or chronic asthma and inflammation (United State patent, 2006). It was successfully used in skin (Gupta et al., 2002; Ehling et al., 2004; Gambichler et al., 2008) and inflammatory cuta-neous diseases (Carroll, et al., 2004; Rubegni et al., 2006; Kymionis et al., 2008), facial tissue transplant (Silverman et al., 2008) and Crohn's disease (Juillerat et al., 2007). It also protected cavernous nerves after crush injury (Valentine et al., 2007). FK506 prevented cadmium induced testicular toxicity in mice (Martin et al., 2007). The role of FK506-binding protein 52 was found essential in uterine reproductive physiology (Yang et al., 2006). Tacrolimus treatment in pregnant rats during tubal transit

period was found not to induce any toxicity (Ramos et al., 2008).

Most of the cytotoxic immunosuppressant drugs were found to possess mutagenic potential and interfered with fertility (Tuner, 2009). Such drugs also induced second-dary carcinomas (Maluccio et al., 2003). Therefore, more toxicity studies are required to evaluate the toxic potential of such drugs. In the current study, the effect of FK506 treatment on meiotic chromosome in testes, epididymal spermatozoa and fertility in male mice were evaluated and the results are presented in this communication.

MATERIALS AND METHODS

Drug product

FK506 was purchased from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

Animal stocks

Swiss albino mice (SWR) home bred, aged 6 to 9 weeks and weighing 23 to 27 g, were used in this study. The animals were maintained under controlled temperature (22 ± 1°C), humidity (50 to 55%) and light cycle (12 h dark and light). They were provided with Purina chow diet (Grain Silos and Flour Mills Organization, Saudi Arabia) and had free access to water (Al-Ashban et al., 2005).

A total of 80 male mice were randomly assigned to different control and treatment groups (five animals in each group). The study under different parameters constituted sub acute (seven days) administration (gavage) of FK506 in saline at doses of 4, 8 and 16 mg/kg/day body weight. The dose selected for the study was the same or multiple of the doses used in different studies on FK506 (Lagoda et al., 2007). The different experimental groups of mice were as follows: (1) untreated control (saline), (2) 4 mg FK506/kg/day, (3) 8 mg FK506/kg/day and (4) 16 mg FK506/kg/day. At the end of the treatment, these groups of mice were set up for evaluation by different parameters.

In the current study, animals were treated (gavage) with relatively higher doses of FK506 because of its bioavailability. The bioavail-ability of enterally administered FK506 was reported to be poor and to prevent treatment failure intravenous administration was preferred, however, sublingual administration of FK506 also provided therapeutic levels in lung, liver and kidney transplantation (Romero et al., 2008).

Meiotic chromosomes

For analysis of meiotic chromosomal aberrations, 5 male mice were used for each dose level and control group. On 19th day from the last treatment day, the mice were sacrificed. The testes were removed and placed in 2.2% isotonic sodium citrate solution. The tunica albugenia was peeled out and somniferous tubules were treated to form a cell suspension. The suspension was centrifuged and the pellet was resuspended in fixative (methanol and acetic acid, 3:1). The chromosomal preparations were made by the air drying technique. The coded slides were stained in Giemsa solution and the spermato-cytes at the diakinesis-metaphase 1 stages were examined for chromosomal aberrations including aneuploids, autosomal univalents, sex-univalents, polyploids and translocations. The significance levels of various meiotic chromosomal aberrations were evaluated by Student's t-test.

Table 1. Effect of FK 506 on testis chromosomes in mice.

Treatment and dose (mg/kg/day)	Meta-phases screened	Percent chromosomal aberrations (Mean ± S.E.)					Translocations		Total chromosomal aberrations (%)
		Bivalents	Aneuploid	Autosoma	Sex univalent	Polyploid	Chains	Rings	
Control (Dist. H₂O)	544	73.98 ± 3.91	10.14 ± 2.10	5.90 ± 1.13	3.51 ± 0.92	6.83 ± 1.09	-	-	26.28 ± 3.02
FK506 (4)	455	64.39 ± 5.34	14.06 ± 3.06	7.69 ± 1.56	5.02 ± 1.09	8.79 ± 2.23	-	-	35.61 ± 4.08*
FK506 (8)	493	52.35 ± 3.12*	17.38 ± 2.04*	11.86 ± 2.05*	6.54 ± 2.24*	11.25 ± 1.6**	1.0 ± 0.6	0.4 ± 0.24	48.07 ± 3.61**
FK506 (16)	577	42.98 ± 6.30**	17.33 ± 2.35*	15.95 ± 3.11**	7.28 ± 1.93**	13.52 ± 2.6**	1.8 ± 1.1	1.6 ± 0.70	57.02 ± 5.92**

*$P < 0.05$;   **$P < 0.01$ (Student's t-test). A total of five mice were used in each group. All groups were compared to the control (distilled H₂O) group.

Sperm abnormality test

Sperm head abnormalities were examined according to the method described earlier (Shah et al., 1991). Five male mice were used in each of the control and test groups. The animals were killed 35 days after the last treatment. The caudae epididymides and vas deferens were dissected in a centrifuge tube containing 3 ml of Krebs Ringer bicarbonate buffer. The sperm solution was filtered through an 80 μm silk mesh to remove tissue fragments and 0.5 ml of the filtrate transferred to a centrifuge tube to which 0.05 ml of 1 percent Eosin was added. The solution was thoroughly mixed and the slides were made by placing one drop of the stained solution on a slide and spreading by three passes of another slide. Coded slides were examined for the abnormalities of sperm head including amorphous, banana shaped, swollen achrosome, flat head, macrocephalic and rotated head. The significance level of abnormal sperms was evaluated by Student's t-test.

Dominant lethal test for fertility and embryonic loss

The Dominant lethal test was conducted according to the method described by earlier researchers. Ten male mice were used for each dose level and control group. Twenty four hours following the treatment, each male mouse was mated to 3 untreated normal virgin females. Thirteen days following the midweek of their first caging and presumptive mating, the female mice were killed. The uterine tract was examined and the numbers of living and dead implants were counted for each pregnant female. From this data base, the following para-meters were evaluated; (1) fertility index was computed as number of pregnant females per number of mated females, (2) the total loss was assessed by comparing the number of live implants in the treated and control animals, (3) pre-implantation loss was determined by com-paring the number of implants per pregnant female in the treatment group and control groups, and (4) post implantation loss; the measure of dominant lethal mutations is referred to the number of dead implants per pregnant female.

RESULTS

The results obtained in the present study on chromosomal changes in germ cells of the male mice treated with FK506 are presented in Table 1. The treatment with FK506 increased the frequen-cy of chromosomal aberrations at all the three dose levels. However, the increase was statis-tically significant for aberrations such as aneu-ploids, autosomal univalents and polyploids at the higher dose levels (groups 3 and 4) when compared with the control. Although, the treat-ment produced some translocations at the higher doses of 8 and 16 mg/kg body weight, but these changes were not found to be statistically significant.

The abnormal sperms observed in mice after FK506 treatment, are shown in Table 2. There was a dose dependent increase in the total abnormal sperms at all the dose levels. However, the increase was statistically significant at the higher dose levels (group 3 and 4) when compared with the control. The frequency of different individual abnormal spermatozoa such as amorphous, banana shaped, swollen achrosome, flat head, macro-cephali and rotated head also increased at all the doses used. However, the increase was statistically significant at the higher doses (8 and 16 mg/kg) when compared with the control.

The data on percent fertility for females mated with FK506 treated males mice is presented in Table 3. The results show that, the pregnancy rate was significantly affected by the treatment at the higher doses (8 and 16 mg/kg) when compared with the control. The total implants per pregnant female (Table 4) were found to be significantly lowered at the higher doses (groups 3 and 4) indicating the impact of FK506 treatment on pre-implantation loss. The numbers of live implants per pregnant female were also significantly reduced at the higher doses of FK506 treatment, when compared with the control, thus, revealing the total loss induced by the treatment. However, the increase in the frequency of dead implants per pregnant female was statis-tically not significant at different doses of FK506

Table 2. Effect of FK506 on epididymal spermatozoa abnormalities in mice.

Treatment and dose (mg/kg/day)	Epididymal spermatozoa abnormalities (%) (Mean ± S.E.)						Total sperms screened N	Abnormal sperms	Abnormal (%)
	Amorphous	Banana-shaped	Swollen achrosome	Flat head	Macro-cephali	Rotated head			
Control Dist.H_2O	0.37 ± 0.03	0.23 ± 0.06	0.42 ± 0.02	0.59 ± 0.05	0.51 ± 0.07	0.36 ± 0.05	6698	164	2.47 ± 0.11
FK506 (4)	0.88 ± 0.47	0.27 ± 0.09	0.37 ± 0.06	0.49 ± 0.16	1.07 ± 0.38	0.47 ± 0.05	5347	162	3.00 ± 0.58
FK506 (8)	1.17 ± 0.39*	1.22 ± 0.42	1.22 ± 0.28*	2.04 ± 0.83*	1.69 ± 0.41	0.81 ± 0.15	4620	376	8.08 ± 2.14*
FK506 (16)	1.54 ± 0.40**	1.92 ± 0.65**	1.27 ± 0.32*	2.10 ± 0.78*	1.83 ± 0.49*	0.95 ± 0.24	4447	427	9.54 ± 2.78**

*$P < 0.05$; **$P < 0.01$ (Students' t-test). Five animals were used in each group. All groups were compared with the control group.
Dist., Distilled.

Table 3. Effect on fertility of FK506 treatment in male mice, mated with untreated female mice.

Treatment and dose (mg/Kg/day)	Number of male mice mated	Number of female mice mated	Total number of pregnant female mice	Percent fertility
Control (Distilled water)	10	30	25	83.33
FK506 (4)	10	30	23	76.67
FK506 (8)	10	30	18	60.00*
FK506 (16)	10	30	17	56.67*

*$P < 0.05$ (Student's t-test).

treatment when compared to the control.

DISCUSSION

The results obtained in the present study are presented in Tables 1-4. FK506 sub acute treatment in mice significantly increased the aberrations in meiotic chromosomes and abnormal spermatozoa in epididymis and vas deferens, indicating it to be a germ cell mutagen. These results are supported by the observed reduction in the fertility, total embryonic loss and pre-implantation loss as observed in the current study. FK506 treatment earlier exhibited higher amounts of micronuclei and reduction in the cytokinesis-block proliferation index (Oliveira et al., 2004). Our findings are substantiated by the results on impairment of spermatogenesis caused by FK506 based immuno-suppressant chemotherapy (Seethalakshmi et al., 1992). Furthermore, FK506 prolonged treatment was found to induce disorders in the seminiferous tubules resulting in spermatogenic damage (Canequim et al., 2009). FK506 treatment was also shown to cause degeneration of sperm cells in epididymis without affecting the production of sperms in the testes and without altering the levels of circulating follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and prolactin (Akbari et al., 2003; Kantarci et al., 2004). Our findings are in agreement with earlier reports suggesting that, chromosomal aberrations in male germ cells might cause changes in sperm head morphology resulting in reduced fertility (Sher et al., 2010), embryonic loss and heritable changes. In the present study, male mice dosed with FK506 (gavage) were also mated with non-dosed female mice to get data on the fertility potential of the male mice. There was no effect on the fertility index, but a decrease in the number of live fetuses associated with implantation loss was noticed. FK506 was earlier reported to induce adverse effects on pregnancy and foetus in female mice indicating its teratogenic nature (Farley et al., 1991). Since most of the mutagens are known to be cyto-toxic and teratogenic; the adverse effects of FK506 reported on pregnancy and foetus and our current observations, might be attributed to its clastogenic

Table 4. Induction of embryonic loss in female mice mated with FK506 treated male mice.

Treatment and dose (mg/Kg/day)	Total number of pregnant female mice	Total implants / pregnant female mice (Mean ± S.E.)	Live implants / pregnant female mice (Mean ± S.E.)	Dead implants/ pregnant female mice (Mean ± S.E.)
Control (Distilled water)	25	11.84 ± 0.65	11.28 ± 0.68	0.56 ± 0.22
FK506 (4)	23	11.26 ± 0.46	10.69 ± 0.59	0.57 ± 0.20
FK506 (8)	18	9.83 ± 0.50	8.78 ± 0.70	1.05 ± 0.28*
FK506 (16)	17	9.88 ± 0.48	8.70 ± 0.80	1.18 ± 0.25*

*$P<0.05$ (Student's t-test).

potential.

Most of the cytotoxic immunosuppressants are known to be pro-oxidant, cytotoxic, mutagenic and induce sexual dysfunction (Oliveira et al., 2004; Rath and Oliveira-Frick, 2009). The cytotoxic and mutagenic functions of such drugs are mostly associated with the induction of cellular lipid peroxidation (Esterbauer et al., 1990). Although, the adverse effects of FK506 observed in the present study simulate to a possible pro-oxidant nature of the drug, however, a large body of evidence suggest that, FK506 acts as an anti-radical (Lupp et al., 2006; Manakova et al., 2006; Wierzbicka et al., 2007; Hisatomi et al., 2008; Sher and Alyemeni, 2010a; Chrazanowska et al., 2010). It decreased oxidative stress by reducing malon-dialdehyde levels, the activity of myeloperoxidase, neu-trophilic infiltration and promoted recovery after caver-nous nerve injury through increased glutathione pero-xidase activity (Dlugosz et al., 2007; Lagoda et al., 2007). FK506 treatment also increased the levels of glutathione in rat liver and kidney (Lagoda et al., 2007) and it inhibited nitric oxide formation (Dlugosz et al., 2007).

In another study, FK506 was found to prevent hepatocellular necrosis, neutrophillic infiltration and en-zyme leakage in hepatic ischemia (Fujishiro et al., 2010). FK506 also down-regulated free radical levels in liver and suppressed cytokine response, tumor necrosis and decreased neutrophil tissue migration (Sher et al., 2010). Cytosolic protein FKBP12 was found to mediate FK506 inhibition of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) transcripts. Some other studies suggested antioxidative properties of FK506 (Lagoda et al., 2007; Valentine et al., 2007; Chrzanowska et al., 2010).

The available literature data are inconsistent on possible pro-oxidant and/or antioxidant effects of FK506, however, FK506 was shown mainly to possess an antioxidant nature where as cyclosporin A showed both pro-oxidant and antioxidant properties depending on the antioxidant capacity of a tissue and cytochrome P450 isoforms present (Lupp et al., 2006; Chrzanowska et al., 2010). The inhibitory effect of FK506 on cytokines also suggested its role in preventing the accumulation of reactive oxygen species and the resulting cellular lipid peroxidation (Hisatomi et al., 2008). Hence, the induction of spermatogenic dysfunction by FK506 treatment obser-ved in the present study may be due to a mechanism other than lipid peroxidation.

Conclusion

The overall conclusion that emerged from the present study is that, the effect of FK506 on spermatogenic dysfunction, fertility and embryonic loss reflects the chromosomal aberrations in germ cells. These aberrations might have been induced due to non-disjunction or the arrest of spindle formation at metaphase stage due to the drug itself or its mutagenic metabolites. The exact mechanism of FK506 induced mutagenicity is not known. However, the inhibitory effect of the drug on FK binding protein (FKBP) might have interfered with the distinct signal transduction pathways in the cells. The interference in the signal transduction pathways due to inhibition of proline rotamase is known to interfere with mRNA, transcription processes and prolife-ration of cells. Further studies are warranted to evaluate the safe dose and duration of therapy to determine the exact mode of action of FK506 induced germ cell toxicity.

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