Long-Term Exposure of Sildenafil Citrate on Sperm Parameters in Rat

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ABSTRACT

Sildenafil citrate (SIL) a phosphodiesterase 5 inhibitor (PDE5I) has been used for long time as a first line oral drug for erectile dysfunction. Though it has beneficial effects on erectile organ it also has some adverse effects in other cells and/or tissues related to reproductive system when exposed to longer duration. The objective of the present study is to evaluate the long term effect of SIL on sperm parameters in Wistar albino rat. The animals are divided into two groups, for group I - rats were treated with saline (vehicle alone) and group - II oral administration of 5 mg/kg b.w. of SIL was administrated orally once in a day for 120 days. At the end of the trial period animals were sacrificed and epididymal sperm were subjected to various analysis. Results showed significant reduction in sperm count, motility, viability and morphologically intact sperm in long term PDE5I exposed animals when compared to control. Acrosomal status and fertility test also showed significant reduction in long term PDE5I exposed animals. The present study clearly indicated that long term SIL has shown to induce alteration in sperm quality and quantity, leading to decline in fertility rate. Indicate that SIL impinge on spermatogenesis as well as epididymal function. Understanding the molecular down-stream events involved in long-term exposure to PDE5 inhibitor can be valuable to supervise on related infertility issues and to suggest corrective measures.

(Key words : Sildenafil citrate, Sperm, Acrosome, Fertility, Rat)

INTRODUCTION

Sildenafil citrate (phosphodiesterase 5 inhibitor- PDE-5I) is the first line oral therapy to treat erectile dysfunction (ED). Both clinical and animal studies revealed that PDE5I has been shown to increase the duration and rigidity of penile erection (Goldstein *et al*., 1998). The main action of sildenafil is the enhancement of the effect of nitric oxide (NO) by inhibiting the cyclic guanosine monophosphate (cGMP) specific PDE5 an enzyme responsible for degradation of cGMP that promotes vasodilatation and achieving penile erection (Boolell *et al*., 1996). It also enhance the sexual activity and increase the post ejaculatory refractory time in both human (Aversa *et al*., 2000) and animal model (Suresh *et al*., 2009). It is a potent and selective inhibitor of the cyclic guanosine monophosphate (cGMP)-specific PDE type-5 enzyme (Ballard *et al*., 1998).

Earlier *in-vivo* and *in-vitro* studies revealed that SIL exposure enhance the sperm motility, viability and capacitation (Purvis *et al*., 2002; Burger *et al*., 2000). Though it is showing beneficial effect on erectile function in both human and animal model (Rendell *et al*, 1999; Bivalacqua *et al*., 2004) Kovanecz *et al*., (2008) found that long term exposure of SIL alter the smooth muscle and collagen ratio in erectile tissue. In consistent with literature our previous study documented that 60 days exposure of SIL significantly increase accumulation of the collagen in penile tissue (Suresh and Prakash, 2011). It was also reported to produce nasal air flow block and obstruction in human subjects who underwent treatment for ED (Kiroglu *et al*., 2006). High doses and long term administration of SIL caused varying degree of cellular neurodegenerative changes like cellular hypertrophy, clustering of cells and intercellular vacuolations in the medial geniculate body of adult Wistar rats (Eweka and Eweka, 2011).

Increased tubular degeneration in testicular epithelium and also reducing the testicular cholesterol and triglycerides with respective increase in the lipid and lipoprotein levels was also reported (Sivasankaran *et al*., 2007).

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Even though it improves the sperm motility when the sperm is exposed to SIL *in-vitro* it also produces premature acrosomal maturation (Glenn *et al*., 2007). SIL treated rat showed reduced fertilization rate and delayed initial embryo development (Glenn *et al*., 2009). However, long term effect of SIL on male reproductive organ is still not clear. In this present study the long term effect i.e. 120 days exposure of SIL on sperm parameters were analyzed.

MATERIALS AND METHODS

Animal Used

Twenty male Wistar albino rats (*Rattus norvegicus*) were used for this study. Male rats of body weight (b.w.) 225~250 g were selected. They were housed individually in separate standard cages and maintained under standard laboratory conditions (temperature 24~28 ℃, relative humidity 60~70%, and 12-hour light-dark cycle) with free access to solid pellet diet and water *ad libitum* throughout the study. The study was approved by Institutional Ethical Committee. Quarantine procedures and animal maintenance was according to the recommendations of Canadian Council Guide to the Care and Use of Experimental Animals (1993) and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines for laboratory animal facility (2003). Experimentations on animal were carried out after subjecting to a quarantine period of not less than 15 days each.

Sildenafil Citrate Administration

After the quarantine periods the animals were divided into two groups group I - treated with saline (vehicle alone) and group - II oral administration of 5 mg /kg b.w. of SIL. The administration was given once in a day for 120 days. The dosage was selected from our pervious study (Suresh *et al*., 2009). At the end of the 120 days animals were sacrificed and the epididymal sperm parameter were carried out.

SPERM ANALYSIS

Sperm Count

Sperm count was done according to the procedure described previously (Suresh *et al*., 2010). Briefly spermatozoa were collect from caudal portion of epididymis, by mincing caudal epididymis with anatomical scissors in 5 ml of pre warmed (35℃) physiological saline, placed in a rocker for 10 min. Supernatant fluid was diluted 1:100 with solution containing 5 g Sodium bicarbonate, 1 ml formalin (35%) and 25 mg Eosin/ 100 ml H_2O. Total sperm was determined with haemocytometer. Approximately 10 µl of diluted sperm suspension was transferred to each counting chamber and was allowed to stand for 5 min and counted under a light microscope at 400 × magnification.

Sperm Viability and Motility

About 20 µl of sperm suspension was mixed with an equal volume of 0.05% eosin-Y and nigrosin. After 2 min incubation at room temperature, slides were viewed by bright-field microscope with 400 × magnification (Nikon, Japan). Dead sperms appeared pink and live sperms were not stained (Suresh *et al*., 2010). Percentage of motile sperm was assessed using graded semi-quantitative scale of 0 to 5 and the spermatozoa were evaluated for the rate of forward movement and graded accordingly, i.e., 0=no movement, 1=sluggish or tail movement alone, 2=intermittent sluggish movement, 3~4=fair & good movement and 5=maximum movement in forward direction.

Morphology and Morphometry

The fixed sperm were smeared on a glass slide and stained with phosphate buffered saline solution of Giemsa (Merck, Germany) (Hafez, 1977). For morphology analysis one hundred sperm were evaluated. Similarly a hundred spermatozoa (intact sperm) per animal were evaluated for their quantifying length of the head and flagellum under the light microscope, using an ocular micrometer scale in 40× magnification, as previously described elsewhere (Suresh and Prakash, 2010).

Cytoplasmic Droplet Containing Sperm

The presences of cytoplasmic droplet containing sperm were determined using the method of Syntin & Robaire (2001). Spermatozoa were assessed for the presence or absence of cytoplasmic droplet, for which minimum of 100-spermatozoa/ animal were evaluated.

Analysis of Sperm Acrosomal Status

Analysis of acrosomal status was done by the method described by Fiscor *et al*. (1983) Briefly, a volume of 100 µl of 2.5% gelatin suspension was smeared in precooled microscope slide and air dried. The slides were washed thoroughly with PBS after fixed with 0.05% of glutaraldehyde for 2 min and stored over night in moist chamber at 4℃. Fifty µl of sperm suspension were smeared on the gelatin coated micro-slide and allowed to dry. Then it was incubated in a moisture chamber at 39℃ for 24 hour. Slides were then stained with coomassie blue for 10 minutes in room temperature. Wash the slide with $D.H_2O$ to remove the excess stain and dry it. Slides were observed under bright field microscope at 200 × magnification. The sperm

with a bright clear zone (hallo) around the head were considered to have good acrosome. The halos were evaluated by measuring the number of sperm cells with or without halo for given sample. About 200 hundred spermatozoa were analyzed for this parameter.

Fertility Test

The fertility test was carried out according to the methods described by Suresh and Prakash (2010). After mating, when spermatozoa were found in the vaginal smear, it was considered as day 0 of gestation. Positive animals were allowed for the full gestation periods for normal delivery and the number of litters were counted and compared between the control and SIL treated experimental groups.

RESULTS

Sperm Analysis

Maintenance of normal concentration of sperm is crucial for the fertilization since reduced sperm count also play a major role in infertility. Concentration of epididymal sperm was evaluated at end of the experiment period (120 days of SIL administration). The sperm concentration was significantly (p<0.01) decreased in the long term sildenafil exposed rats. No significant changes were observed in the morphometry study in all the experimental groups.

Motility of the sperm is a very important parameter to evaluate the fertility (fertilizing) ability of the organism. Effect of long term SIL exposure on epididymal sperm motility was done at the end of 120 days. The data depict that there was significant (0.001) decline in motility in SIL exposed rat when compared with control rat (Table 1).

Table 1. Illustrate the sperm parameters (count, motility, viability and sperm with cytoplasmic droplet), a- control and *-p<0.001.

S.No.	Parameter	Control	Sildenafil citrate
	Sperm viability (%)		
1.	Live	98±1.23	69.48±7.23 a*
	Dead	2±0.89	31±7.24 a*
2.	Motility (%)	98±2.15	53 ±3.73 a*
3.	Count (×10^6)	285±11.59	212.5 ±13.25 a*
4.	Morphology (%)		
	Normal	96±2.12	66±11.07a*
	Abnormal	4±0.35	44±1.32 a*
5.	Cytoplasmic droplets	1.67±0.82	35.67±6.50 a*

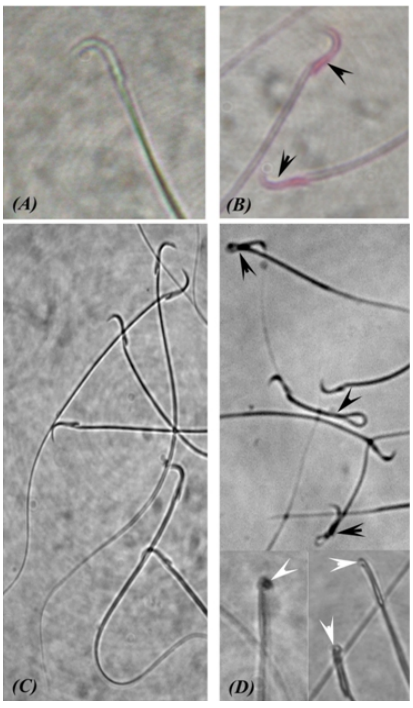

Fig. 1. Illustrates the viability and morphology of epididymal sperm control (A, C) and long term sildenafil citrate exposed group (B, D). The long term sildenafil citrate exposed rat showing more dead sperm (black arrow in B) self coiling spermatozoa (black arrow-D) and head abnormality (white arrow-D).

The viability of epididymal sperm was analyzed after the 120 days exposure of SIL by eosin-Y and nigrosin staining. Data showed significant reduction in percentage of viable sperm (p<0.001) with concomitant increase in the dead sperm in the long term sildenafil exposed rats when compared with control rats (Table 1).

Morphology and Morphometrical Analysis

Morphological analysis showed wide degree of abnormality in long term sildenafil treated rat sperm such as defects in the head (microcephalic, bicephalous, amorphous, and acephalic), neck and tail when compared to the control (Table 1 and Fig. 1). No significant alteration was observed in morphometrical study in light microscopy.

Cytoplasmic Droplet

Cytoplasmic droplet study showed significant (p<0.001) increase of the sperm that had cytoplasmic droplets in long term sildenafil exposed rats (41±2.35) when compared to control rats (2.59±0.43). These cytoplasmic droplets were located at various levels in the sperm; tail remnant was more in number. Observations are summarized in table 1.

Acrosomal Status Test

Long term effect of the SIL exposure on epididymal

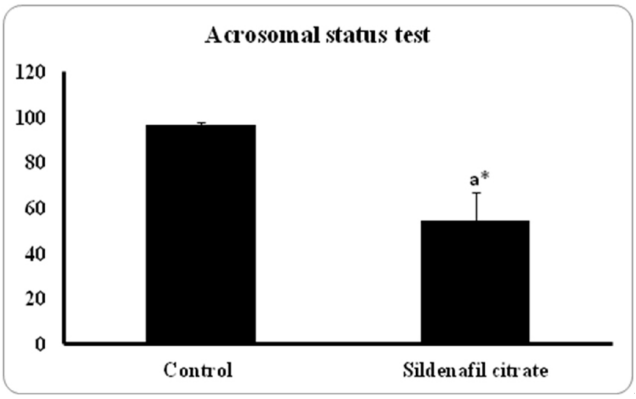

Fig. 2. Illustrates the acrosomal status of t control and long term sildenafil citrate exposed groups. Each value indicates the mean± SEM of (n=6) animals, a- control and *–p<0.001.

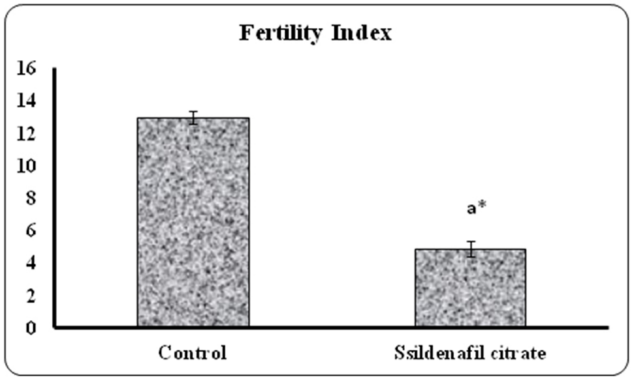

Fig. 3. Showing the fertility index of control and long term sildenafil citrate exposed groups. Each value indicates the mean± SEM of (n=6) animals, a- control and *–p<0.001.

sperm acrosomal status was analyzed by simple gelatin digestion methods. There was significant (p<0.01) reduction in gelatin digestion (sperm with halos) was observed in long term sildenafil exposed rats when compared to control (Fig. 2).

Fertility Test

Effect of long term sildenafil exposure on fertility capacity was assessed by counting the number of litters in female rats mated with sildenafil exposed rat. Data clearly indicates that SIL exposed rats showed significant (p<0.001) reduction in number of litter when compared to control (Fig. 3).

DISCUSSION

Structural and functional integrity of sperm is very essential for achievement of the fertilization and good quality of progeny. Impairment of sperm quality and quantity plays a major role on male infertility. In the present study reduced sperm count motility and viability was observed in long term (120 days) SIL exposed rat, which indicates that SIL have toxic effect on spermatogenesis and/or the spermiogenesis at the testicular level leading to increased production of defective sperm.

Previous reports from human studies showed that use of SIL can improve sperm motility and activate acrosomal reaction in short term treatment (Burger *et al*., 2000; Mostafa, 2007). Present data showed significant reduction in acrosomal functional status test in long term SIL treated rat, which demonstrate that long term exposure might inhibit acrosomal formation or induce the premature acrosomal reaction (Glenn *et al*., 2007). In consistent with pervious study, present result also indicate that long term SIL exposure significantly alter the fertilizing capacity of the sperm (Glenn *et al*., 2009). Association between reduced acrosomal functional status and decline in fertility signifies the role of long term influence of SIL on rat sperm.

Increased amount of immature and abnormal sperm in SIL treated rats might be due to long term effect of sildenafil on Sertoli cell function leading to impaired spermatogenesis or spermiation which can produce immature or sperm with cytoplasmic droplets (Suresh and Prakash, 2010). Normally in epididymis, sperm with cytoplasmic remnant were removed in their course in epididymal tubules (Cosentino and Cockett, 1986). Though analyzes of head and flagellum size showed no significant alteration in SIL exposed rats, increased morphological defects and cytoplasmic remnant in sperm after long term SIL exposure in rat indicate that it also affect epididymal function.

The significant increase in sperm with cytoplasmic droplet can impair motility and reduce fertilizing capacity, as it might be an extra weight for the sperm that make prolong travel to reach the ampullary part of fallopian tubes for fertilization (Cooper, 2011). In addition remnant cytoplasmic droplet might expose the sperm to go for osmotic burst in female reproductive tract leading to immotile or sluggish motility. Data revealed that SIL can implicate infertility effect.

Observed changes in sperm parameters suggested that long term SIL intake can produce severe alterations in testis and epididymis and induce sperm damage, resulting in infertility (Glenn *et al*., 2009). In recent decades both old and young men are using PDE5 inhibitor to enhance penile erection for good sexual life. Though SIL could produce beneficiary effect in short term but in long term administration can increase the risk of infertility, indicated by the reproductive toxicity in epididymal sperm observed in present study. Thus SIL impinge on spermatogenesis as well as epididymal function. Understanding the molecular down-stream events involved in long term exposure to PDE5 inhibitor through basic and clinical research can be valuable to su-

pervise on this infertility issue to propose corrective measures.

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