

EFFECT OF NIMODIPINE ON MALE REPRODUCTIVE FUNCTIONS IN RATS

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Abstract : Nimodipine, a dihydropyridine calcium channel blocker, was administered orally using two different doses (40 mg and 60 mg/kg/day) to rats. Both short term (2 weeks) and long term (6 weeks) effects of the drug were observed. The drug administration resulted in a marked decrease in sperm density, sperm motility and acrosomal reaction. Zona-pellucida penetration by the sperm obtained from drug-treated animals was significantly lower when compared with sperm from normal animals. Nimodipine stimulated Ca^{2+} ATPase activity in isolated plasma membrane of rat spermatozoa. In conclusion, short term and long term administration of nimodipine has deleterious effect on male reproductive functions in rats.

Key words : nimodipine

rats male reproduction

INTRODUCTION

Sperm motility capacitation and acrosome reaction are the pre-requisites for the successful oocyte fertilization. Intracellular calcium is a key regulator of sperm function because it plays a critical role in sperm motility and acrosome reaction. As a pre-requisite to fertilization, sperms undergo an acrosome reaction which is mediated in part by increasing permeability to Ca^{2+} , with an attendant rapid, massive Intracellular Ca^{2+} accumulation (1). It is well documented that only acrosome reacted spermatozoa can bind to and pass through the zona-pellucida

of the ovum (2).

Calcium channel blocker, verapamil has been shown to inhibit Ca^{2+} influx and consequently the acrosome reaction (3). Present study was undertaken in male rats to elucidate the in vivo effects of long term (6 weeks) and short term (2 weeks) treatment of calcium channel blocker, nimodipine, on spermatozoal functions-motility, density and Ca^{2+} ATPase activity. Further, studies of in-vitro acrosomal reaction and zona pellucida penetration test with spermatozoa of experimental animals were carried out to see the ultimate effect of nimodipine on fertilizing ability of spermatozoa.

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METHODS

Healthy adult male albino rats (*Rattus norvegicus*) with a body weight between 175 to 250 gms were obtained from the institute animal house. Animals were acclimatized in the laboratory conditions for a period of one month before starting the experiment and were fed pellet chow and water ad libitum.

The acclimatized animals were randomly divided into following three groups consisting of 16 rats in each group.

Group I : Control (Vehicle - 2 ml of 0.5% Tween-80)

Group II : Nimodipine 40 mg/kg (in 2 ml of 0.5% Tween-80)

Group III : Nimodipine 60 mg/kg (in 2 ml of 0.5% Tween-80)

Groups (I-III) were further subdivided into two groups (a & b). Group a and group b received treatment for 2 wks and 6 wks respectively. The dose of nimodipine was selected after conducting pilot study using 3 different doses (20 mg, 40 mg & 60 mg) of nimodipine.

Nimodipine (Nimodipine, Torrent Pharmaceutical Ltd., Ahmedabad, India) was suspended in 2 ml of 0.5% Tween-80 and administered daily orally by tube feeding for respective periods.

Healthy adult female albino rats weighing 150-200 gms were used for studies related to zona pellucida penetration.

After completion of the treatment rats were sacrificed by overdose of anaesthetic agent and the following parameters were assessed.

Sperm density

Spermatozoa were collected by flushing the vas deferens and epididymis in 2.0 ml of normal saline and diluted 20 times with normal saline. Sperm count was done in a Neubauer's haemocytometer chamber under light microscope. Sperm count/ml was calculated as $A \times 20 \times 10^6 / \text{ml}$, where A is the average mean of outer four square (4).

Sperm motility

Motility was checked for progressive (both hyperactivated and moderate) forward movements according to the WHO Laboratory manual procedure (4). In order to calculate percent motile sperms, at least 100 sperms were checked in 10 different fields (5). The motility count was motile vs immotile sperms.

Acrosome reaction (*in vitro*)

Acrosome reaction was performed according to the method of Yanagimachi (6). After collecting semen by flushing the lumen of the vas deferens with 0.9% NaCl, spermatozoa were washed once by centrifugation (400 g, 10 min.) and resuspended in 0.9% NaCl. Aliquants (0.1 ml) were added to 0.9 ml of the Minimum Capacitation Medium (MCM) in sterile polystyrene tubes with caps (Falcon

Plastics, No. 2003, Oxnard, California). The tubes were placed horizontally on a shaker (Yankee, Clay Adams, Parsippany, New Jersey), rotating at 60 strokes per minute in an incubator at 37°C with air as the atmosphere.

At various times during an incubation, small aliquants (0.01 ml) of the sperm suspension ($3.5 \times 10^7/\text{ml}$) were taken out and mounted between slide and cover slip. At least 100 spermatozoa were examined to estimate the percentage of cells without a visible acrosomal cap within the motile sperm population by phase-contrast microscopy $\times 320$ (Olympus, Japan).

Effective Acrosome reaction (AR%) was calculated by using the following formula (7).

$$\% \text{ AR} = \frac{\text{Motile sperms without acrosomal cap}}{\text{Total no. of motile sperms in the whole population}} \times 100$$

Zona-Pellucida Penetration Test (*in vitro*)

Zona-pellucida penetration test was performed with live spermatozoa obtained from both drug treated and control female rats (8).

Superovulation of rats

Immature female rats were obtained from the institute's central animal house and acclimatized to laboratory conditions for one month. Adult female rat (150–200 gms) were induced to superovulate by injecting intramuscularly 30 I.U. of pregnant mare's serum gonadotropin. After 48 to 72 hours 40 I.U. of human chorionic gonadotropin

(HCG) was injected intramuscularly. Seventeen hours after the injection of HCG, oviduct was flushed with 0.1 M phosphate buffer saline, pH 7.54. Cumulus oophorus was removed by rinsing the infusate 2–3 with MCM which had been previously passed through millipore filter.

In vitro fertilization

After the completion of experiment rats were sacrificed at 10 a.m. after overnight fasting. Spermatozoa were collected in 2.0 ml of normal saline from vas deferens and distal portion of the epididymis. Sperm suspension was prepared immediately before mixing with MCM. Appropriate amount of MCM was added to get a sperm density of $3.5 \times 10^7/\text{ml}$ in sperm suspension. The suspension was divided into small aliquants to get $5\text{--}7 \times 10^6$ sperms/ml.

10–20 μl of the prepared sperm suspension ($5\text{--}7 \times 10^6$ sperm cells/ml) were added to 100–200 μl of oocyte suspension (~ 100 oocytes) on a watch glass and were mixed thoroughly with a needle and covered with mineral oil, incubated at 37°C for 2 hours. After 2 hours 10 μl of the sperm-oocyte suspension was taken and mounted on a slide with coverslip and observed under a phase contrast microscope. The slides were observed for percent zona-pellucida penetration by the spermatozoa (8).

Assay of Ca^{2+} ATPase activity

The Ca^{2+} ATPase activity was estimated by the method of Quigley and Gotterer (9).

ATPase activity was measured under two conditions :

- (i) In presence of Ca^{2+} , Na^+ and K^+ (total ATPase).
- (ii) In presence of Ca^{2+} , Na^+ , K^+ and ouabain (ouabain insensitive ATPase).

The (Na^+ - K^+) stimulated ATPase activity was obtained by subtracting ouabain insensitive ATPase from total ATPase. Ouabain insensitive ATPase was taken as Ca^{2+} ATPase activity.

Reagents

- (A) 75mM Tris-HCl buffer (pH 7.1).
- (B) 50 mM adenosine triphosphate (ATP). The pH of this solution was set at 4.1 and the solution was always made fresh.
- (C) 10 mM ouabain
- (D) 50 mM CaCl_2
- (E) 200 mM KCl
- (F) 1.2 M NaCl
- (G) 2.5 M KCl
- (H) 2.5 M perchloric acid

Procedure

- A mixture of 0.4 ml of 75 mM Tris-HCl buffer, 0.1 ml of 50 mM ATP, 0.1 ml of 75 mM CaCl_2 , 0.1 ml of 200 mM KCl and 0.1 ml of 1.2M NaCl were taken in small glass test tubes.

- For total ATPase, 0.1 ml distilled water and for ouabain insensitive ATPase 0.1 ml of 10 mM ouabain were added separately.
- The tubes were perincubated for 5 min. at 37°C .
- Suitably diluted enzyme (0.1 ml) was added in each tube making the final volume to 0.1 ml. The tubes were stirred and incubation carried out for 30 min. at 37°C .
- The reaction was terminated by adding 0.25 ml of 2.5M perchloric acid in each tube and immersed in ice bath for several minutes.
- These precipitates were then coprecipitated with 0.25 ml of 2.5M KCl. The tubes were replaced in ice bath and then spun for 10 min. at a low speed to obtain a clear supernatant.
- An aliquot of the supernatant was then assayed for inorganic phosphorus. The estimation of inorganic phosphorous released by the hydrolysis of ATP was done by the method of Chen et al. (10). Reagent blank was also run along with water blank.
- Specific activity of the enzyme was expressed as nmol/mg Pr/min.

Statistical analysis

Values are expressed as mean \pm SEM. The analysis of variance (ANOVA) was used for sperm density and Ca^{2+} ATPase activity. The

Chi-square (X^2) test was applied for non-parametric data like sperm motility, acrosomal reaction and zona-pellucida penetration. Values having $P < 0.05$ were considered statistically significant.

RESULTS

Sperm density

Nimodipine (40 mg and 60 mg/kg/day) significantly ($P < 0.05$) decreased sperm density in both 2 weeks and 6 weeks treated groups (Table I). Decreased in sperm density was more pronounced in 6 weeks treatment.

Sperm motility

Both 2 weeks and 6 weeks treatment with nimodipine (40 mg and 60 mg/kg/day) showed significant ($P < 0.05$) inhibitions of sperm motility (Table I).

Acrosome reaction

Significant decrease in acrosome reaction was seen with nimodipine 60 mg/kg, both in 2 week and 6 weeks treatment groups, whereas nimodipine 40 mg/kg produce significant decrease in acrosome reaction only in 6 weeks treatment group (Table II).

TABLE I: Effect of nimodipine on spermatozoal functions in rats.

	Control group (Vehicle treatment)		Test group (Nimodipine treatment)			
	2 wks	6 wks	40 mg/kg		60 mg/kg	
			2 wks	6 wks	2 wks	6 wks
Sperm Density (Million/ml)	95.4±1.81	94.56±6.55	79.93±3.85*	47.88±2.84*	66.0±3.83*	40.63±4.22*
Sperm motility (%)	70.81±3.57	71.13±4.36	47.25±1.98*	40.5±1.38*	49.38±2.01*	39.0±3.79*
Zona-Pellucida Penetration (%)	47.63±7.66	44.38±8.05	32.62±10.59*	29.25±6.52*	20.5±8.21*	24.5±6.41*
Ca ²⁺ ATPases activity (n moles/mg pr/min)	13.96±0.47	13.71±0.79	17.93±0.63*	21.63±1.04*	24.83±0.73*	26.53±0.82*

Values are expressed as Mean ± SEM, n=8, * $P < 0.05$ as compared to control.

TABLE II: Effect of nimodipine on acrosomal reaction in rats (*in vitro*).

Group	Acrosomal reaction (%)					
	2 wks treatment			6 wks Treatment		
	1 hr	2 hrs	3 hrs	1 hr	2 hrs	3 hrs
Control	49.25±4.42	63.0±2.83	70.13±4.54	45.25±1.99	61.5±2.18	69.75±2.07
NIMODIPINE						
40 mg/kg/day	43.75±1.67	53.63±1.76	60.5±2.11	30.13±2.12*	39.63±1.83*	52.38±1.40*
60 mg/kg/day	34.63±3.36*	45.75±5.28*	54.5±3.34*	34.13±2.44*	45.38±2.95*	51.63±2.71*

Values are expressed as Mean ± SEM, n=8; * $P < 0.05$ as compared to control.

Zona-Pellucida penetration

Nimodipine (40 mg and 60 mg/kg) significantly ($P < 0.05$) inhibited the zona-pellucida penetration capability of sperm after 2 weeks and 6 weeks treatment.

Ca²⁺ ATPase activity

The specific activity of Ca²⁺ ATPase was found to be significantly stimulated in sperm plasma membrane of drug treated animals (Table I). Nimodipine (40 mg and 60 mg/kg) showed a significant increase in Ca²⁺ ATPase activity after both short and long term treatment.

DISCUSSION

It is well documented that intracellular calcium within a narrow range of concentration has a regulatory role in sperm motility and acrosome reaction (6). Indirect evidence suggests that increased permeability of the spermatozoal membranes to Ca²⁺ is a crucial component of capacitation that allows the subsequent occurrence of acrosome reaction and motility activation (11). The present study was planned to see the effect of calcium channel blocker, nimodipine on spermatozoal functions.

In vivo administration of nimodipine resulted in decreased sperm density which may be due to partial arrest of spermatogenesis. The arrest of spermatogenesis can be attributed to the inhibition of CAMP phosphodiesterase

system (12) or low levels of prolactin (13) and androgen (14) following the treatment of animals with nimodipine. The present set of data indicates a marked inhibition of sperm motility which could be due to low levels of ATP content (15) and is in agreement with the findings of Juneja et al (3). This decrease might be due to detrimental effect of excessive intracellular calcium on the mitochondrial energy coupling system (16) thereby inhibiting sperm motility. The adverse effect of an excessively high intracellular calcium concentration by nimodipine may also result in inhibition of acrosomal reaction (6) and could account for the reduced capability of nimodipine treated spermatozoa to penetrate the zona-pellucida of superovulated rat ova which would have a deleterious effect on fertilizing potential of rat spermatozoa.

In several cell system, Ca²⁺ ATPase located in the plasma membrane helps to maintain low concentration of intracellular calcium by pumping this ion out of the cell (17) and responsible for the fine tuning of Ca²⁺ levels inside the cells. Juneja et al. (3) have shown that following calcium channel blockers treatment, there was an excess of intracellular calcium levels which interact with plasma membrane Ca²⁺ ATPase system and ultimately stimulates the membrane enzyme activity to restore the intracellular Ca²⁺ content to normal concentration. Our results show an increase in membrane Ca²⁺ ATP activity following nimodipine treatment which may be due to increased intracellular Ca²⁺ concentration.

Although it is not always feasible to extrapolate the results obtained in animals to those in humans yet the findings of the present study demonstrate the in vivo effects of nimodipine on the male reproductive physiology.

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