SEARCH FOR NOVEL ANTI-FERTILITY AGENT BY MONITORING *IN VITRO* METABOLIC INHIBITION, CELL MOTILITY AND CELLULAR INTERACTIONS OF NIFEDIPINE ANALOGUES

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ABSTRACT

Current family planning measures predominantly target a female clientele, with relatively few significant developments in male fertility regulation. At present, only effective methods for contraception in men are those that prevent sperm transport, such as condoms and vasectomy. Thus, in an attempt to synthesize non-hormonal, safe, reversible and oral male contraceptive, we have used nifedipine as a prototype molecule. Nifedipine is a calcium channel blocker and popular anti-hypertensive drug. Its reversible anti-fertility effect is a well-known side effect. In order to develop male oral contraceptive, we have synthesized four analogues; *m*-hydroxy (D_5), *m*-chloro (D_6), *p*-nitro (D_7), *p*-methoxy (D_8) aryl 1, 4-dihydropyridine derivative of nifedipine and monitored their effect on sperm motility and metabolic activity. To highlight their mechanism of action on sperm function through membrane interaction, we have studied their molecular level interactions with model membrane using NMR and DSC technique. One of the synthesized analogues (D_5) showed promising results.

Keywords: Anti-fertility; Nifedipine; model membrane; DPPC; NMR; DSC.

INTRODUCTION

Effective regulation of human fertility has global consequences in terms of resource depletion, pollution and poverty. Current family planning measures predominantly target a female clientele with few significant developments in male fertility regulation. The last two decades have witnessed a gathering interest in the development of safe, reversible, non-hormonal orally active methods of contraception for men[1].

Some of the commercial drugs have shown significant effects on male fertility. For instance, hormonal drugs, anti-malarial, anti-convulsants and opioids have all been reported to have anti-fertility effects on male rats[2]. Interestingly, dihydropyridine (e.g. nifedipine), L-type calcium channel blocker, is known to cause reversible anti-fertility, by interacting at calcium mobilization process – calcium entry through L-type voltage gated calcium channel. Calcium ion has been shown to be a primary determinant of sperm cell function, including progressive motility, hyper-activated motility, capacitation, and acrosome reaction. Accordingly, a number of Ca^{2+} permeable channels and transporters have been reported to be present in sperm. Functional studies have also documented the presence of voltage-gated Ca^{2+} channels in mammalian sperm.

We have previously reported a detailed study on nifedipine and its analogues where sperm motility, metabolism, lipid peroxidation and lipid composition have been monitored as markers of the sperm function/ fertility[3]. It is well documented that para substitution of 1, 4-dihydropyridine has less cardiotherapeutic (anti-hypertensive) activity. In our previous study, we have synthesized and evaluated ortho and para substituted analogues of nifedipine which have shown promising results towards their anti-fertility effects. In the present study, we have further explored the effect of various substituents and their positions on anti-fertility action. This led to the synthesis of meta-hydroxy (D_5), meta-chloro (D_6), para-nitro (D_7) and para-methoxy (D_8) aryl 1, 4-dihydropyridine analogues of nifedipine (Fig. I). These have been synthesized using the classical Hantzsch reaction scheme[4], by modifying the carboxyl ester chain on dihydropyridyl nucleus along with substitution on aryl nucleus. We have evaluated anti-fertility activity of the analogues by monitoring their effect on sperm motility and metabolic profiling of viable sperms using ^{13}C NMR technique. In addition, membrane lipid peroxidation (LP) to assess the damaging effect of the synthesized analogues

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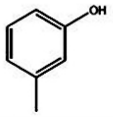
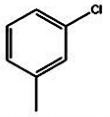
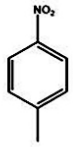
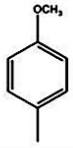
Compound	R	R_1
D_5	C_2H_5	
D_6	C_2H_5	
D_7	C_2H_5	
D_8	C_2H_5	

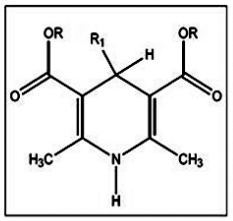


Fig. 1: Molecular structure of nifedipine and its analogues

on sperm membrane integrity, motility and functionality of sperm cells have been evaluated. In order to highlight the mechanism, we studied the intermolecular interactions with L-α-diapalmitoyl phosphatidylcholine (DPPC) model membrane by DSC and NMR techniques.

MATERIALS AND METHODS

Materials

Nifedipine was a gift sample from Unichem Ltd. 3-Hydroxy benzaldehyde, 3-chloro benzaldehyde, 4-nitro benzaldehyde and anisaldehyde was purchased from S.D. Fine-Chem Ltd. DPPC was purchased from Sigma Chemicals Co., U.S.A. All other solvents used for synthesis were of LR grade.

METHODS

Synthesis of nifedipine analogues

For the synthesis of dihyrdropyridine compounds, Hantzsch reaction method was used. The synthesized derivatives are shown in Fig. I. This involves a multi-component condensation of an aldehyde (1 mol) with a 1, 3- dicarbonyl compound (2 mol) and ammonia by refluxing with methanol. Four different analogues of nifedipine have been synthesized by modifying the aryl nucleus and increasing the alkyl ester side chain without altering the dihydropyridine nucleus. The detailed synthetic approach and characterization is given in supplementary data and scheme is shown in supplementary Fig. S1.

DSC experiments

DSC measurements were carried out on differential scanning calorimeter VP-DSC (MicroCal, Northhampton MA, USA). The samples were degassed under vacuum before being loaded into the reference and sample cells. A scan rate of 10^0C/h was employed. Data was analyzed with the software ORIGIN provided by MicroCal. All the experiments were carried out in the temperature range 20^0C to 60^0C. Three samples of each data set were collected. Repeated scans for the same sample were generally superimposable.

NMR experiments

NMR experiments were recorded on BRUKER AVANCE 500 and 700 MHz NMR spectrometers. 2D-COSY, NOESY, TOCSY and ROESY were recorded using standard pulse programs[5–7]. ^{31}P and ^{13}C NMR experiments were carried out with a relaxation delay of 2 sec. and broadband proton decoupling. The NMR data was processed using Bruker Topspin 2.1.

Sample Preparation for NMR and DSC experiments

Multilamellar vesicles (MLVs) were prepared using standard procedure[8], wherein the desired quantities of DPPC and synthesized molecules were dissolved in chloroform. The solvent was then evaporated with a stream

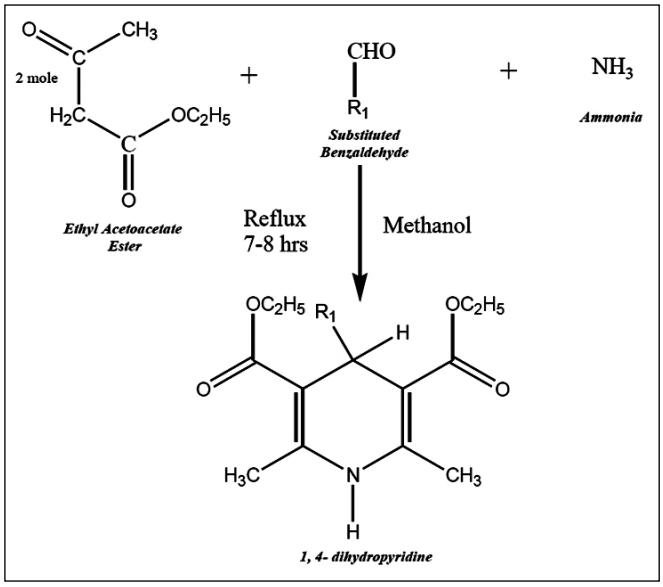


Fig. S1: Synthetic scheme of analogues of nifedipine based on Hantzsch reaction

of nitrogen gas so as to deposit a thin film on the walls of the container. The last traces of the solvent were removed using vacuum for 2 h. The lipid film was hydrated with the required amount of D_2O; this was then incubated for half an hour in a water bath at 50^0C with repeated vortexing. The lipid concentrations were maintained at 100 mM for the NMR and 50 mM for the DSC experiments. Unilamellar vesicles (ULVs) were prepared by sonicating the above dispersions with a Branson sonicator (Model 450) at 50% duty cycles till the solution was optically clear.

Determination of MLV-drug binding

Binding constants were determined by the centrifugation method[9]. Optical density of 100 µM solution of the drug molecules was measured using spectrophotometer at a wavelength range of 220-400 λ. MLVs were prepared by varying lipid concentration systematically from 0.25mg/mL to 2.0 mg/mL and fixed drug concentration of 100 µM. The resulting solutions were incubated for 2 hours at 50^0C and subsequently transferred into ultracentrifuge tubes. Separation of liposomes from the aqueous phase was achieved by centrifugation at 40,000 rpm for 2 hours. The drug concentration in the supernatant was determined by measuring the optical density. The fraction of drug bound to liposomes was determined from the difference. The drug-liposome apparent binding constant (k) was analyzed using the double reciprocal plot. A plot of 1/ (fraction bound) vs. 1/ (lipid concentration) yields a straight line with slope 1/k.

EVALUATION OF ANTI-FERTILITY ACTIVITY BY METABOLIC PROFILING

Cell sample preparation

NMR measurements were conducted in real time on viable sperm under anaerobic conditions[10]. Cells were collected from cauda region of goat epididymis by gentle mincing and tweezing in Dulbecco buffer. Tissue pieces were removed by allowing the cell suspension to settle for 5 minutes. The cells were washed, made into a pellet by centrifugation, and then suspended in an appropriate quantity of buffer to attain desired concentration.

Metabolic profile measurement

The ^{13}C NMR experiments were carried out on Bruker 500 MHz spectrometer using 25,000 Hz spectral width, 60° flip angle and 2 seconds relaxation delay with power gated broadband proton decoupling. Dulbecco buffer with 10% D_2O was used for NMR field-frequency locking. Glucose labeled with ^{13}C at C-1 position was used as the substrate for glycolytic reaction. ^{13}C NMR spectra monitored as a function of time show a decrease in glucose signal (substrate consumption) intensity and an increase in lactate signal (buildup) intensities. The progress of glycolysis has been monitored by directly measuring glucose consumption and lactate production with time[11–13]. The effect of nifedipine, its analogues on the metabolism of sperm cells was compared by monitoring the lactate signal build up with time and thus estimating their glycolytic inhibitory potency.

Motility analysis

The effect of nifedipine and analogues on sperm motility was measured using the Sander-Cramer test[14]. Concentration of the cell was adjusted by using Dulbecco buffer (control) or with 2.5 mM drug solution[11]. This sample (50 mL) was placed on a slide and covered with a cover glass (18 mm x 18 mm) and at least six microscopic fields were examined. Each slide was evaluated twice. Motility data was collected at two time intervals i.e. after one hour and after two hours of incubation. Sperm motility inhibition was expressed as a percentage scale.

Lipid peroxidation

Lipid peroxidation (LP) was assessed by formation of thiobarbituric acid reactive substance (TBARS), malondialdehyde[15]. Thiobarbituric acid (TBA) reagent (10 mL composed of 0.35% w/V thiobarbituric acid and 15% w/V trichloroacetic acid in 0.25N hydrochloric acid) was prepared. Sperm cell samples were incubated with varying concentrations of drugs/analogues to induce peroxidation and to release malondialdehyde which is thiobarbituric acid reactive substance formed within the cell due to lipid peroxidation. TBA reagent was added equally to all the samples, incubated for 10 minutes at 100 ^0C, cooled and centrifuged at 1500 rpm for 10 minutes and the absorbance of supernatant recorded at 532 nm.

RESULTS

Binding studies with MLVs

The fraction of nifedipine and synthetic analogues bound to lipid is calculated. A plot of the inverse of the fraction of drug *vs* the inverse of lipid concentration is linear. The apparent binding constants measured for different molecules are nifedipine - 0.0851M^{-1}, D_5 - 0.0191 M^{-1}, D_6 - 0.0587 M^{-1}, D_7 - 0.1604 M^{-1}, D_8 - 0.0083 M^{-1}. The extent of binding is in the order of D_7 > Nifedipine > D_6 > D_5 > D_8. These results indicate that these molecules bind to the MLVs with variable degree of affinity based on their structural differences.

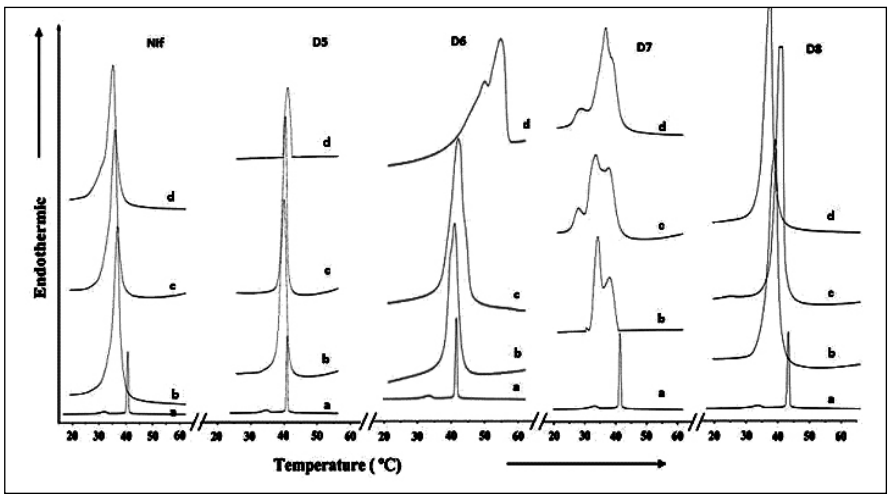


Fig. 2: DSC plots of DPPC (50mM) incorporated with (Nif) nifedipine, D_5, D_6, D_7, and D_8. The additives: lipid molar ratios are (a) 0:100, (b) 1:10 (c) 1:5 and (d) 1:2

DSC studies

The thermotropic aspect of drug-lipid interaction was monitored using DSC technique by examining the changes in the melting temperature (T_m) of the lipid bilayer and the shape of the DSC trace[16–18]. Fig. 2 shows DSC curves of lipid bilayers prepared from DPPC incorporated with varying concentrations of nifedipine, and its analogues. At zero drug concentration (trace a), multilamellar bilayers of DPPC show a pre-transition at 33.82^{0}C and a main transition (T_m) at 41.26^{0}C due to the mobility of the polar choline head group and the alkyl chains, respectively. On addition of varying concentrations of nifedipine and its analogues into DPPC bilayer, at 1:10 molar ratios, there is a change in both pre-transition and main-transition temperatures. In case of nifedipine, the T_m shifts to a lower value and broadens. The pre-transition peak completely vanishes, broadens nearly to baseline. These results indicate that nifedipine imparts fluidity to the bilayer[19]. Fig. 2 (D_5-D_8) are DSC curves of DPPC bilayers incorporated with D_5, D_6, D_7 and D_8 respectively with increasing concentrations of analogues. Noticeably, large change in the pre-transition temperature is observed in all the cases. In the case of D_5, with increasing concentration, the shape of endotherm is retained, pre-transition peak completely broadens and T_m peak becomes broad and shifts to a lower temperature. In case of D_6, lower concentration shows similar behavior as that of D_5 while at higher concentration (at 1:2 molar ratio), abnormal shift and broadening is observed which is an indication of the formation of isle like structure[20]. D_8 shows a shift in T_m to a lower value by 3-4 ^{0}C with significant broadening. All these DSC endotherms highlight that D_5, D_6, and D_8 impart fluidity to the bilayer. On incorporation of D_7, i.e. para-nitro substituted analogue, at molar ratio 1:10 (peak b) the pre-transition peak is abolished and T_m shifts to a lower value (3.36 ^{0}C). The appearance of shoulder to the T_m and larger broadening indicates larger perturbation to the membrane structure. It also indicates that D_7 could be intercalated within lipid polar head by hydrogen bonds, electrostatic interaction or by penetration into the alkyl chain region leading to a large disruption to the lipid bilayer.

These results thus confirm that the type of substituents in nifedipine molecule play an important role in the stabilization of membrane.

NMR experiments

^{1}H NMR spectra of the synthesized analogues have been assigned using 2D COSY spectra (figure not shown). Comparison of the ^{1}H NMR spectra of DPPC ULVs and drugs incorporated into ULVs does not show any significant change in the chemical shift of DPPC resonances.

The 2D NOESY spectrum contains a wealth of information on intermolecular interactions as well as the conformation of the molecules in the lipid bilayer. The intermolecular NOEs observed between the lipid and nifedipine, and its analogues have been presented in Supplementary data#. A few NOEs could not be unambiguously analyzed due to spectral overlap. The data indicate that all the molecules show intermolecular interactions with lipid bilayer with some variations. Nifedipine, D_5, D_6 and D_8 show NOEs with both head as well as tail ($(CH_2)_n$-CH_3)) region of the lipid bilayer. This is more likely of hydrophobic interaction with lipid bilayer chain and hydrophilic interaction with head group. In the case of D_5 number of additional intermolecular NOEs are observed as compared to those for other analogues as shown in Fig. 3. In contrast, D_7 shows preferential binding to hydrophobic tail region of lipid bilayer. This indicates that D_7 gets buried in the hydrophobic core of the membrane altering the bilayer phase to a larger extent. These results support the observations made in binding studies as well as those in DSC experiments.

To get insight into the nature of the intermolecular interactions between nifedipine, and its analogues with the lipid membrane, ^{13}C NMR experiments on these molecules incorporated into the lipid bilayer have been carried out. The spectra of the plain DPPC bilayer, nifedipine, and

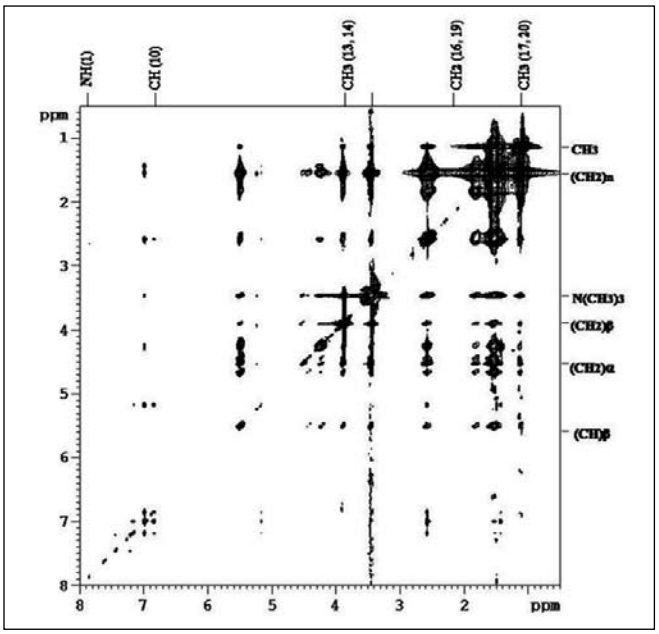


Fig. 3: Representative 2D NOESY spectrum of DPPC unilamellar vesicles incorporated with D_5 [1:5 D_5: lipid molar ratio]. The experiment was carried out at 323K with a mixing time of 400ms

its analogues show sharp signals. On incorporation of nifedipine and its analogues in ULVs, the ^{13}C signals arising from the lipid and the drug show differential broadening. This is because different parts of drug bind to the lipid bilayer with different affinities. This differential broadening can be split into three categories: signals that remain sharp but are shifted, those that are broad and shifted and those that are broad beyond detection.

In case of nifedipine and its analogues D_6, D_7, on incorporation into the bilayer all the signals are broadened almost to baseline (Fig. 4 [trace Nif, D_6, D_7]). The broadening of the signals arises due to an exchange at intermediate time scale between the bound and the free form of these drugs. Due to the broad nature of the signals it is not possible to measure both the spin lattice relaxation time (T_1) and the spin–spin relaxation time (T_2) which was a measure of the overall tumbling behavior and segmental motion of the molecule[21]. In the fast tumbling range, both the T_1 and T_2 are large, of the order of a few seconds. However, these molecules lose their mobility and become strongly bound to lipid bilayer resulting in loss of motional freedom. This also leads to an increase in the motional ordering of the lipid acyl chain and the head group. The analogues D_5 and D_8 (Fig. 4 [trace D_5, D_8]) show an average binding characteristic, where the molecules are in a free motion/ fast exchange.

The results further indicate that binding of these molecules to DPPC is greatly dependent on the nature of the derivative. D_5 and D_8 show an average binding characteristic. On the other hand, nifedipine, D_6 and D_7 are strongly bound to the lipid bilayer and lose their motional freedom, as indicated by a complete broadening of the signals to baseline. This indicates that the drug is highly immobilized in the lipid bilayer due to strong hydrophobic interaction.

^{31}P NMR spectroscopy is sensitive to local motions and the orientation of the phosphate group in the membrane, making it a well-suited tool for monitoring structural changes and detecting polymorphism in model membrane. The ^{31}P NMR resonance line shape is determined by the chemical shift anisotropy (CSA) of the phosphate group coupled with the molecular motions near the head groups[22].

Study of varying concentrations of nifedipine and analogues D_5, D_6, D_7, D_8, incorporated into lipid bilayers shows that the ^{31}P NMR line shape is not affected seen in Fig. 4 and the bilayer features remain intact. However,

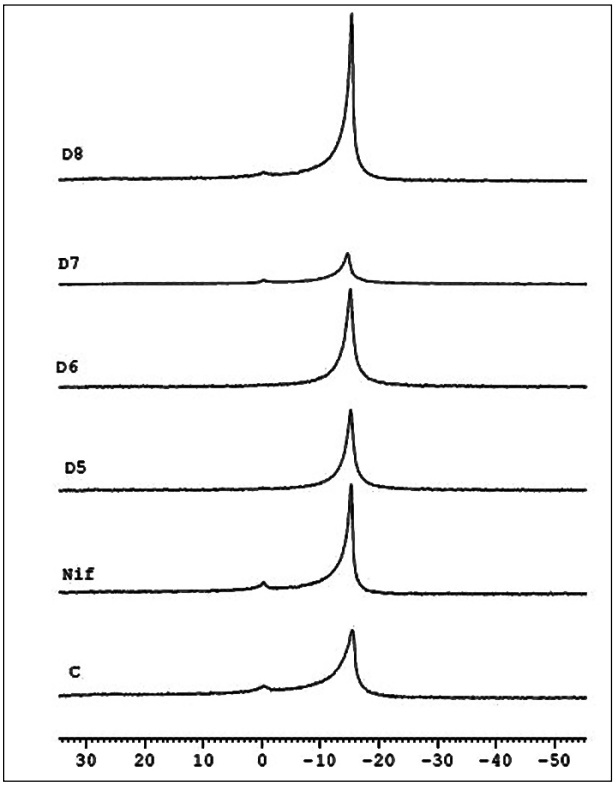


Fig. 4: ^{31}P NMR spectra of (c) DPPC (100 mM) multilamellar vesicles. Nif, D5, D6, D7 and D8 are spectra of DPPC multilamellar vesicles incorporated with respective drug and analogues (1:5 additive: lipid ratio) All experiments are at 323K

the extent of broadening of main peak at -20ppm varies for different analogues, indicating their binding differences.

Cell metabolism

Cell metabolism can be checked by monitoring the ^{1}H, ^{13}C and ^{31}P signals arising from the low molecular weight compounds present in the cells[18]. It is particularly profitable to use ^{13}C labeled substrates. When cells are fed with labeled substrate, the label appears at the corresponding carbon atom in various metabolites. In the current study, sperm cells have been incubated with glucose labeled with ^{13}C at C-1 position, as substrate. ^{13}C NMR spectra, monitored as a function of time, show decrease in glucose (α and β isomers) signal and increase in lactate signal (arising from C-3 methyl) intensities. Experiments performed on spermatozoa incubated with different drug molecules under investigation, suggest that the rates of glucose consumption and lactate generation changes monotonically with an increase in the drug concentration as shown in Fig. 5 Low level of lactate production is a direct indication of metabolic inhibition. As compared to control sample, metabolic inhibition is observed maximum for D_7 amongst all the analogues.

Sperm motility

Effect of nifedipine and its analogues has been studied on sperm cell motility. Results indicate that these molecules render spermatozoa immotile to different extents, as shown in Fig. 6. In one hour, nifedipine causes about 80% decrease in motility and after two hours almost complete decrease in motility is achieved. In case of D_7, in the first hour, decrease in motility is about 40% and thereafter a complete reduction in motility is achieved within 2 hours. D_5, D_6 and D_8 have relatively slow but significant effects (~ 60% to 80% decrease) on motility.

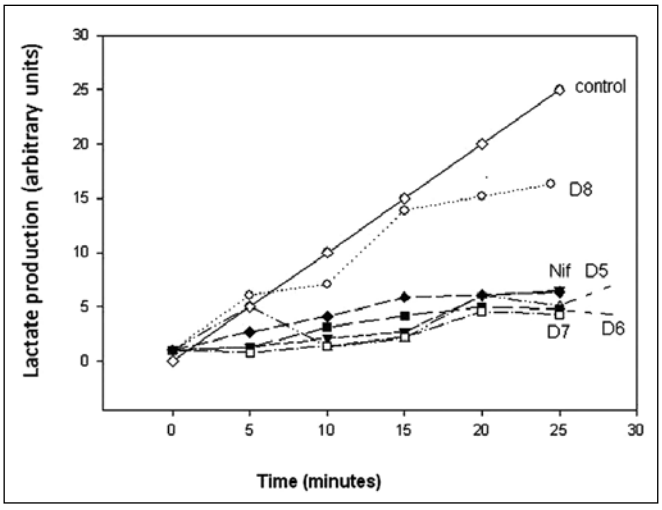


Fig. 5: Lactate production by spermatozoa obtained from goat epididymis with time. Cells are incubated with nifedipine (Nif), nifedipine analogues (D_5-D_8) and control (cells without drug)

Lipid peroxidation

Lipid peroxidation (LP) can be defined as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds due to free radicals. Membrane lipids mainly comprise of phospholipids, and are susceptible to lipid peroxidation as the fatty acids of phospholipids get oxidized by free radicals. Motility of spermatozoa depends on the integrity of the mitochondrial sheath, of which phospholipids are major components; LP may produce detrimental effect on motility and survival of spermatozoa. LP decreases the membrane fluidity, changes the phase properties of the membranes and decreases electrical resistance. Also, cross-linking of membrane components restricts mobility of membrane proteins. Malondialdehyde (MDA) is one of the lipid peroxidation products that reacts with thiobarbituric acid

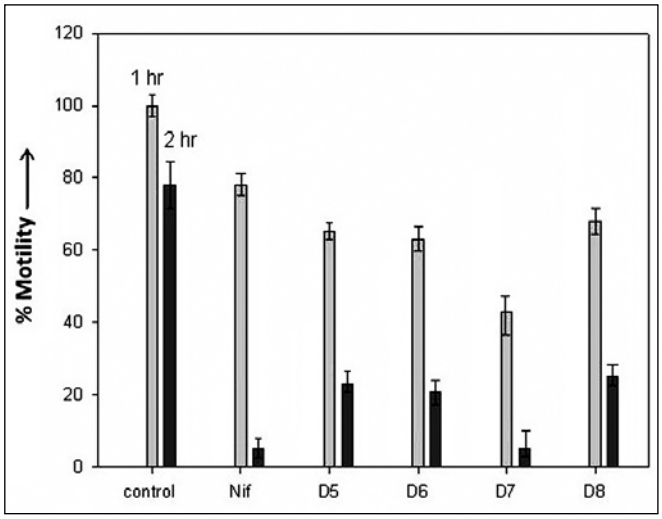


Fig. 6: Percent sperm motility in cells obtained from the cauda region of goat epididymis in presence of nifedipine (Nif) and nifedipine analogues (D_5-D_8), calculated after 1 and 2 h. Percent motility of control sample after 1 hour has been taken as 100

to form a fluorescent red adduct which can be measured spectrophotometrically. Under normal conditions, certain amount of autoperoxidation continues to occur in the cells. Therefore, normal spermatozoa (control sample) also show some amount of absorbance due to the MDA present. We have monitored the damage caused to the spermatozoa by monitoring the lipid peroxidation induced by the drugs/analogues with due consideration to the natural autoperoxidation of the cells as shown in Fig. 7. The MDA absorbance indicates that in the presence of

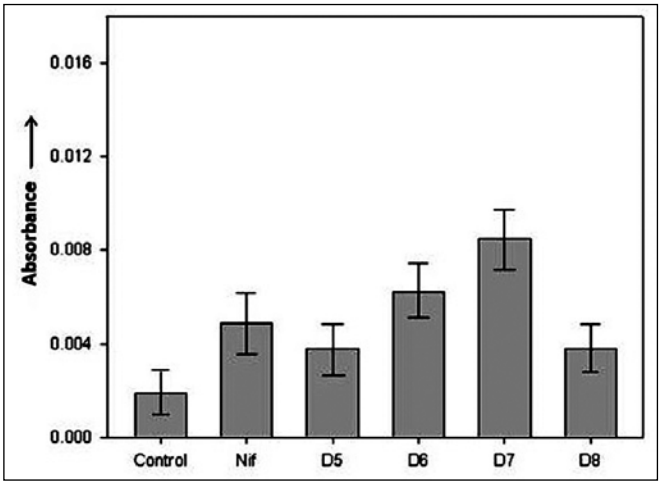


Fig. 7: Absorbance measuring malondialdehyde produced in spermatozoa in presence of (Nif) nifedipine and its analogues (D_5, D_6, D_7, & D_8). Control indicates malondialdehyde produced due to autoperoxidation

D_7 analogue, generation of MDA is large as compared to nifedipine and other analogues. This indicates that D_7 may be altering the membrane integrity of the cells to a larger extent as compared to nifedipine and other analogues. D_5, D_8 had very less damaging effect on cells as seen by decrease in the amount of malondialdehyde generated and lower absorbance values. The overall LP results interpreted as $D_7 > D_6 >$ Nif $> D_5 > D_8$.

The overall interpretation of the present study indicates that the nifedipine analogues act differently with respect to their anti-fertility action. This is achieved by firstly altering the metabolism which directly affects the cell motility, which is the responsible factor for the cells to be fertile. Secondly, these drugs affect the membrane fluidity as well as architecture thereby changing membrane dynamics. This in turn affects the binding of these molecules to the interior of cell membrane resulting in cell damage with regard to the fertility. The NOESY experiments specify that the analogue D_7 may be binding to the interior of the lipid bilayer membrane, disturbing the strong hydrophobic interactions between the lipid molecules. On the other hand, the broadening of peak and lowering of transition temperature in the DSC trace demonstrates that both the size and packing of bilayer is altered, and the system becomes disordered. Thus, the molecule affects the cell motility drastically. On the other hand, the DSC and ^{31}P NMR results highlight that D_5, D_6 and D_8 analogues of nifedipine do not disturb the bilayer phase of the MLVs. These results are further supported by ^{13}C NMR results on *ex-vivo* metabolism where D_7 is found to inhibit lactate production in a much more effective manner than nifedipine. At the same time LP results indicate that D_7 causes membrane damage to a larger extent thereby decreasing the motility of the sperms. D_5 on the other hand shows lesser cell damage similar to nifedipine. This effect of D_5 can contribute towards reversible antifertility activity.

DISCUSSION

Sperm membrane integrity is vital for the process of fertilization. Loss in vitality can be result of immobility of sperm cells or might be due to membrane damage.

Nifedipine shows its reversible anti-fertility effect and causes cell aging by hampering motility, affecting the membrane damage to smaller extent. Similar effects are observed in case of D_5. On other hand D_7 causes cellular injury to a larger extent. This lipid damage affects the membrane integrity which may result in irreversible cell damage. Therefore, D_5 in line with nifedipine can be a potential antifertility agent for future developments and testing.

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