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Research Article

ADRENERGIC ANTAGONIST PROPRANOLOL AS A NOVEL, EFFECTIVE SPERMICIDE: AN NMR STUDY

SUDHA SRIVASTAVA[a] AND EVANS COUTINHO[b]

[a]National Facility for High Field NMR, Tata Institute of Fundamental Research, Mumbai – 400005, India, [b]Bombay Collage of Pharmacy, Kalina, Mumbai-400098, India. Email: sudha@tifr.res.in

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ABSTRACT

Spermicides exert antifertility effect upon spermatozoa as these cells pass through the female genital tract. Considerable interest has been shown on the anti-AIDS potential of nonoxynol-9, a most widely used spermicidal agent. Sperm cells are known to possess components with receptor-like characteristics that can interact with β-adrenergic antagonists. Thus drugs which are able to block β-adrenergic receptors are likely to interfere with sperm motility. In order to investigate the participation of an adrenergic system in regulating sperm motility in mammals, we have carried out a comparative study on the effect of some β-adrenergic blockers and known spermicidal agent nonoxynol-9 on human and goat spermatozoa. Results indicate that both racemic (DL) and the D-enantiomer of propranolol affect sperm motility and metabolic activity in a dose-dependent fashion. The spermicidal action depends upon its local anesthetic properties rather than its β-blocking potential. The D-enantiomer, unlike the racemic DL is devoid of the effects of β-blockade and thus may be a useful drug. Moreover, a combined effect of propranolol and nonoxynol-9 indicates that these compounds interact in a complimentary manner in their ability to inhibit sperm function. A significant enhancement of the spermicidal action of propranolol is observed in Ca^{2+} free medium.

Keywords: Spermatozoa, NMR, Motility, Metabolism, Propranolol, Spermicides,.

INTRODUCTION

Human sperm is a potential model for pharmacological and toxicological research. Spermicides are biologically an obvious way of interrupting human fertility. There are three classes of spermostatic and spermicidal agents that are widely reported. These are surface active agents, enzyme inhibitors and sulfhydryl binding compounds[1]. The contribution of vaginal spermicides to contraception is becoming more widespread[2] but there has been a reduction in the number of products available over the past two decades[3]. Advantages of spermicides include safety, availability, simplicity, convenience, and acceptability. In addition, they are appropriate for short term use or for use in conjunction with other methods.

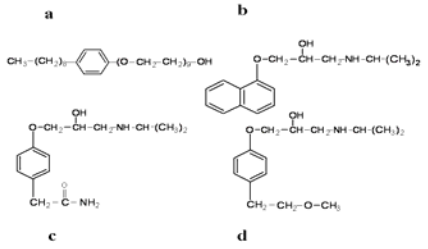

Fig. 1: Molecular structure of a) Nonxynol-9, b) Propranolol, c) Atenolol, d) Metoprolol.

Unfortunately, their failure rate compared with other forms of contraception and the need to apply them shortly before intercourse limit their usefulness. Hence there is an urgent clinical need to research novel safe, effective contraceptives that can prevent unwanted pregnancies and also protection against sexually transmitted diseases (STDs) such as the human immunodeficiency virus (HIV) or *Chlamydia*[4] and other pathogenic organisms.

Spermatozoa can also be immobilized or killed by targeting lipid rafts, lowering pH, or oxidative damage[1,5]. Detergents are surface active agents that kill spermatozoa and inactivate enveloped viruses such as HIV and herpes simplex virus (HSV) by disturbing the lipid bilayer at the cell surface[6]. The most widely available spermicide is the detergent nonoxynol-9 (N9) (Fig. **1(a)**). N-9 is a non-ionic surfactant that is used as an ingredient in a variety of vaginal contraceptives and in various cleansing and cosmetic products. This compound acts by disrupting the sperm plasma membrane, causing rapid immobilization and cell death[7,8]. It is active against HIV *in vitro* and also prevents transmission of simian immunodeficiency virus (SIV) in rhesus macaques *in vivo*[9]. Although it was at one time widely promoted as a protective against sexually transmitted infections including HIV, subsequent studies have shown that it can in fact *increase* the risk of infection by damaging the physical barriers of the rectum or vagina, especially if used frequently[10, 11]. This has essentially stimulated the search for alternative spermicidal agents.

Both α and β-adrenergic receptors have been identified on human sperms[12, 13]. It has been demonstrated that drugs able to block β-adrenergic receptors interfere with sperm motility[14]. Adrenergic monoamines possibly modulate sperm motility by both a calcium-dependent and a cyclic nucleotide-dependent mechanism. Effect of β-adrenergic antagonists on fish sperms indicate that propranolol affects fish sperm motility and causes vesicle formation in sperms[15].

Propranolol (Figure 1(b)) is a β-blocker that is used to treat tremors, angina, hypertension, heart rhythm disorders, etc[16]. It is administered as the racemate, however the D-isomer has the β-blocking activity while the L-isomer has membrane stabilizing effect. Propranolol also posseses local anesthetic activity of short latency and fairly long duration. It is known to inhibit sperm function/motility and sexual dysfunction (impotence)[17]. These properties make it an ideal candidate for development as a potential novel spermicidal agent.

In order to investigate the participation of an adrenergic system in regulating sperm motility and metabolic activity in mammals, we have investigated the effect of some β-blockers like propranolol, atenolol and metoprolol on human and goat spermatozoa. Also their effects have been compared to the known spermicide, N9.

MATERIALS AND METHODS

Chemicals: Propranolol, atenolol, metoprolol, nonoxynol-9, 1-^{13}C glucose were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were of AR grade. Tyrode medium (pH 7.2) and glucose (0.1% w/v) was used as a buffer and fuel for cellular metabolism.

NMR: ^{13}C NMR and ^{31}P NMR experiments were carried out on a Brüker AVANCE 500 MHz NMR spectrometer. ^{13}C chemical shifts were measured with respect to sodium 3-trimethylsilyl-(2,2,3,3-D)-propionate (TSP) and ^{31}P chemical shifts with respect to phosphoric acid. In both the cases, a 10 Hz exponential multiplication factor was applied prior to Fourier transformation. Buffer with 10% D_2O was used for NMR field-frequency locking. NMR measurements have been conducted in real time on viable sperms under anaerobic conditions.

Cells: The study was approved by the Ethics Committee and informed consent statement was obtained from the patients. Semen samples were collected from patients consulting at KEM Hospital Parel, Mumbai. Spermatozoa were separated from seminal plasma by 3 cycles of centrifugation at 500 x g for 5 min in 2ml of Tyrode medium and were finally resuspended in the same medium to attain the desired concentration. Sperm concentration per milliliter was determined using a haemocytometer. The concentration of motile cells in washed sperm preparations was assessed by counting at least 100 cells in a 10µl aliquot at x250, with the aid of a grid on an eye-piece graticule. A final sperm concentration of $1x10^6$ cells/ml was used in all experiments. Cells with motility higher than 70% were used.

Goat cells were obtained from the cauda region of the epididymis. Cells were collected in Tyrode buffer, washed, centrifuged, suspended in buffer to attain a concentration of 1×10^6 cells/ml.

Motility analysis: The effect of drug molecules on sperm motility was tested using the Sander-Cramer test[18]. Semen samples were previously incubated with Tyrode medium (control) or with the required quantity of drug solution for 30 minutes. 50µL of this sample was placed on a slide and covered with a cover glass (18mm×18mm) and at least six microscopic fields were examined. Each slide was evaluated twice. The sperm motility was expressed on a percentage scale.

Metabolism: For both ^{13}C and ^{31}P NMR experiments, cells were incubated with the requisite concentration of drugs and incubated for 30 minutes. In ^{13}C NMR experiments, 1-^{13}C glucose was used as a substrate for the glycolytic reaction. Spectra (24 transients) were recorded with time and the mid-time for each experiment was taken as the corresponding experimental time. The intensity of the signal was measured by peak integration (area under the curve) using inbuilt Brüker software. The NMR signals of glucose (α+β anomers; substrate consumption) and that of lactate (buildup) were monitored over an initial 3 hour period. The glucose (α+β anomers) intensity data with time was fitted to a first-order rate equation (decaying) to determine the rate for substrate consumption, whereas for lactate a rising exponential equation has been used for the calculation of the rate of generation. All data were analysed by ANOVA and Bonferroni tests and expressed as mean ±SEM (n=8). Values were considered significantly different if $P<0.05$.

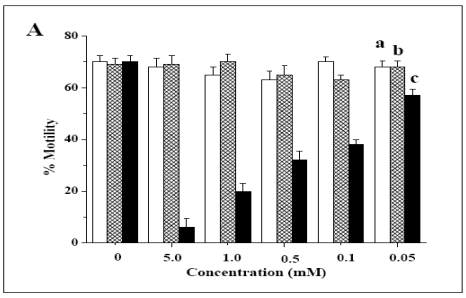

Fig. 2a: Percent sperm motility in cells obtained from goat epididymis in presence of increasing concentration of drugs.

a) atenolol, b) metaprolol c) propranolol. All data have been analysed by ANOVA and Bonferroni tests and expressed as mean ±SEM (n=8). Values are considered significantly different if $P<0.05$.

RESULTS AND DISCUSSION

Figure 2 (A) shows the concentration-dependent inhibition by three drugs propranolol, atenolol and metoprolol on goat sperm motility. The propranolol concentration that decreases sperm motility to 50% of control (EC_{50}) is 0.3 mM. Atenolol and metoprolol do not show any effect on sperm motility. Figure 2 (B) shows the % sperm motility from fertile men in presence of propranolol (5 mM) for different case samples studied. Amongst the six cases presented, the percent motility decreases from 85% to 95% of the control samples in presence of maximum propranolol concentration of 5 mM. However, results vary from case to case, as certain factors vary from one individual to other.

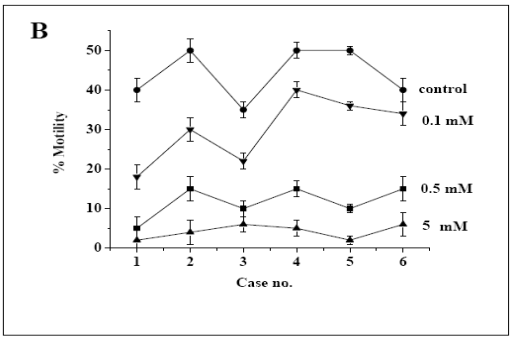

Fig. 2b: Percent sperm motility in cells obtained from fertile men in presence of varying concentration of propranolol for different case samples studied.

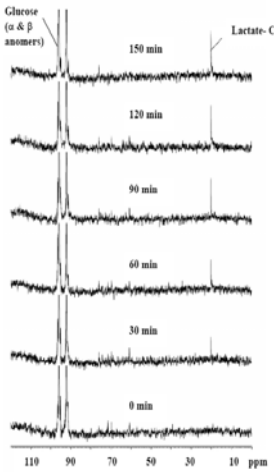

Fig. 3: Time course of lactate production by goat spermatozoa fed on 1-^{13}C glucose.

When 1-^{13}C glucose was added to the cells strong signals from the two isomers of glucose (α and ®) are observed.

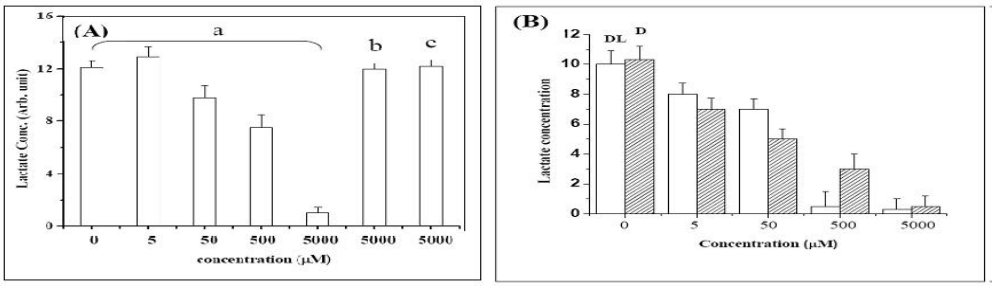

Fig. 4: Lactate production (arbitrary units) by the spermatozoa obtained from goat epididymis incubated with different concentrations of drugs.

(Panel A) a) Propranolol, b) atenolol, c) metoprolol. (Panel B) Empty bars are DL-propranolol, filled bars are D-propranolol. All data has been analysed by ANOVA and Bonferroni tests and expressed as mean ±SEM (n=8). Values are considered significantly different if $P<0.05$.

Carbohydrates and fat are common fuels for motility in spermatozoa. Fatty acids can only be utilized aerobically, while carbohydrate can also be used under anaerobic conditions, but has the disadvantage of producing an approximately 18-fold lower yield of ATP per mol glucose. Under anaerobic conditions, lactate is the main end-product in the spermatozoa of vertebrates and most invertebrates. Therefore, both ATP and lactate can be used as a parameter to monitor the functioning of sperm which can be further correlated to motility and thereby fertilizing ability. Figure 3 shows the time course of lactate production by goat spermatozoa fed on 1-^{13}C glucose. When 1-^{13}C glucose is added to the cells strong signals from two isomers of glucose (□□and β) are observed. As the metabolism progresses with time, these signals decrease in intensity with a consequent build up of lactate which is being monitored by its $^{13}CH_3$-methyl. Figure 4(A) shows the lactate production by the cells incubated with different concentrations of propranolol. It is observed that the rate of lactate production goes down with increasing concentration of propranolol. On the other hand atenolol and metoprolol at the highest concentration used, did not show any inhibitory effect on cell metabolism. This indicates that the β-blockers atenolol and metoprolol do not suppress sperm activity.

Similar experiments have been carried out with D-isomer of propranolol and compared with the racemate DL-propranolol (Fig. 4(B)). It is observed that D-isomer is also effective in suppressing sperm motility, despite the fact that D-propranolol does not possess the ability to block β-receptors[19-21]. These observations imply that the basis of propronolol's spermicidal action may depend upon its local anesthetic properties rather than its β-blocking potential. These results are further supported by earlier reports where sperm immobilizing activity of three class I antiarrhythmic drugs, quinidine, procainamide, mexiletine, one adrenoceptor blocking drug, labetalol and one calcium channel blocking drug, diltiazem was measured with a trans-membrane migration method. In all these cases, the local anesthetic effect was identified as the underlying mechanism responsible for inhibition of sperm motility[14].

^{31}P NMR is a very important tool to monitor high energy metabolites in intact cells[22]. The resonance assignments for various ^{31}P metabolites present in sperm cells are shown in Figure 5 (a). When the cells are pre-incubated with varying concentration of propranolol, the production of these molecules decreases (Figure 5 (b)) or completely inhibited at higher concentration (Figure 5 (c)). However, atenolol and metoprolol do not show a significant amount of effect even at higher concentrations as seen in Figures 5 (d) and (e) respectively. These observations further imply that the basis of propronolol's spermicidal action depends upon its local anesthetic properties rather than its ®-blocking potential.

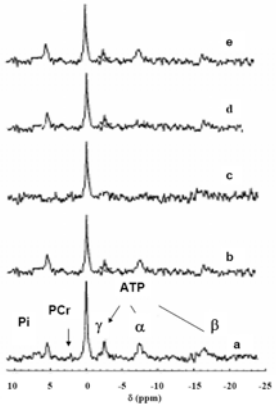

Fig. 5: ^{31}P NMR spectra (202.5 MHz) of spermatozoa obtained from goat epididymis undergoing glycolysis

(a) in Tyrode medium (initial pH 7.2), containing 1% (w/v) glucose.

After 30 minutes incubation with b) DL-propranolol (0.5 mM), c) DL-propranolol (5 mM), d) atenolol (5 mM) and e) metaprolol (5 mM). Spectral parameters used are 1s relaxation delay, 10μs pulse width corresponding to 60° flip angle and 10 kHz spectral width. Chemical shifts have been reported with respect to a phosphoric acid signal. Resonance assignments are shown in (a).

It has been well recognized that the potencies of local anesthetics are determined by their lipid solubilities[23]. The sperm membrane is composed of lipoprotein. It is likely that the extent of adsorption of drugs on the sperm membrane is determined by their lipid solubility and thus the immobilizing effect on the sperm.

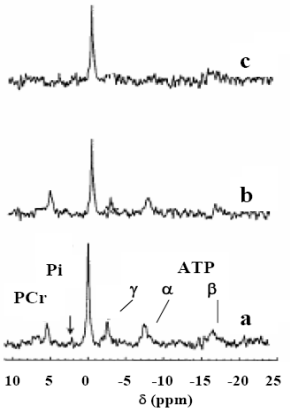

Fig. 6: ^{31}P NMR spectra (202.5 MHz) of spermatozoa obtained from goat epididymis undergoing glycolysis

(a) in Tyrode medium (initial pH 7.2), containing 1% (w/v) glucose.

After 30 minutes incubation with b) DL-propranolol (0.5 mM), c) Nonoxynol-9 (0.5 mM). Spectral parameters used are 1s relaxation delay, 10μs pulse width corresponding to 60° flip angle and 10 kHz spectral width. Chemical shifts have been reported with respect to a phosphoric acid signal. Resonance assignments are shown in (a).

The question now arises is how does the effect of propranolol compare with known antifertility drug(s)? Nonoxynol-9 (N9, a surfactant microbicide) is a non-ionic surfactant used as an ingredient in a variety of vaginal contraceptives and in various cleansing and cosmetic products. There have been increased incidence of HIV infection in nonoxynol-9 users during clinical trials[10, 11]. In spite of its potent microbicidal activity *in vitro*, N-9 not only failed to protect against HIV but also increased the incidence of this infection in users[10, 11]. This anomaly was largely attributed to the strong detergent nature of N-9 that caused a pro-inflammatory reaction in the vagina, resulting increased susceptibility to HIV. Liver toxicity in animals when nonoxynol-9 was injected intraperitoneally is also reported[24]. These observations have stimulated the search for alternative spermicidal agents.

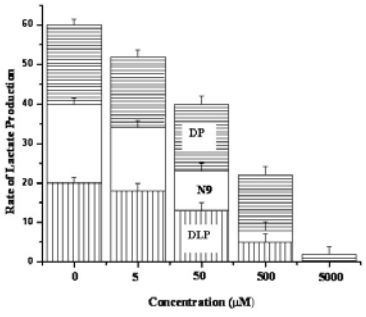

Fig. 7: Rate of lactate production by the spermatozoa obtained from goat epididymis incubated with different concentrations of drugs.

To determine the rate for lactate generation a rising exponential equation has been used for the calculation. DP) stands for D-propranolol, DLP stands for DL-propranolol and N9 for Nonoxynol-9. All data has been analysed by ANOVA and Bonferroni tests and expressed as mean ±SEM (n=8). Values are considered significantly different if $P<0.05$.

In view of these reports we have carried out ^{31}P NMR experiments to monitor the cell energetics in presence of both the drugs (Figure 6). Results indicate that N9 is more effective than propranolol. In Figure 7 the effects of D-propranolol, DL-propranolol and N9 have been compared. In the first column, the three different blocks are the controls where no drug has been added. In the subsequent columns, the corresponding blocks represent the increasing concentration of the added drug, which are in sequence DL-propranolol (DLP), nonoxynol-9 (N9) and D-propranolol (DP). We observe that at 500 µM drug concentration, the respective rate of lactate production in presence of DP, N9 and DLP is only 65%, 20% and 25% of that of control. This indicates that the metabolic inhibition is in the order N9>DLP>DP. DP shows least inhibitory effect. A 10 times higher concentration (5000 µM) of DP is required to attain the same order of inhibitory effect as produced by DLP and N9. These results thus indicate that racemic propranolol is more effective in suppressing sperm motility than the dextro isomer.

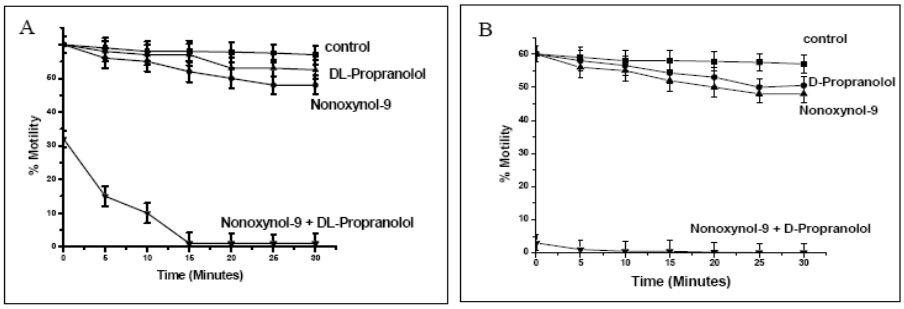

Fig. 8: Effect of propranolol and nonoxynol-9 and their combined effect on sperm motility (%) in cells obtained from goat epididymis.

A) DL-propranolol, B) D-propranolol. All data has been analysed by ANOVA and Bonferroni tests and expressed as mean ±SEM (n=8). Values are considered significantly different if $P<0.05$.

To investigate the combined effects of these two spermicidal agents, a concentration of each compound was chosen that caused only a low inhibition of sperm motility (Figure 8). For DL- propranolol at a concentration of 50 µM no significant effect was observed even after 20 minutes. Nonoxynol-9 at a dose of 50 µM was seen to exhibit a significant effect. However, the combined application of both compounds each at 50 µM concentration, the motility was reduced to 50% within 1 min of addition, and was completely abolished after 15 minutes of incubation. In case of D-propranolol, a higher concentration of 500 µM in conjunction with 50 µM of N9 was seen within 1 min of addition to reduce sperm motility, which was reduced to zero after 15 min of incubation.

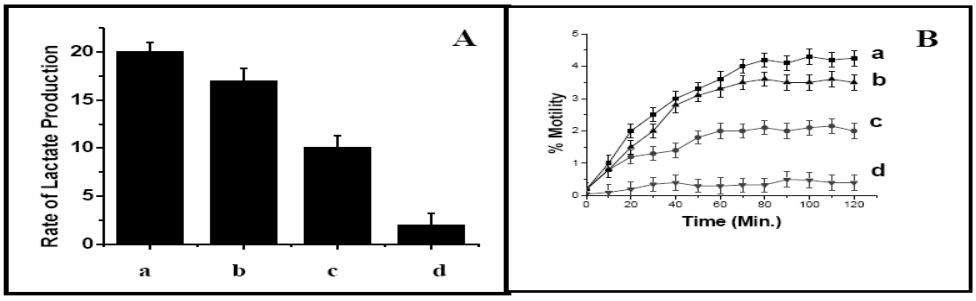

Fig. 9: A) Metabolic activity (lactate production) and B) % motility of cells in Ca^{+2} free and complete medium.

a) cells in complete medium, b) cells in Ca^{+2} free medium, c) cells incubated with 5 mM DL-propranolol in complete medium and d) cells incubated with 1 mM DL-propranolol in Ca^{+2} free medium.

These results thus indicate that propranolol and nonoxynol-9 work in a synergistic manner to inhibit sperm motility. The basis for this complementary effect is unknown. However, this observation probably reflects the fact that these compounds affect different aspects of membrane function.

The capacity of N9 to inactivate HTLV-II125r makes a strong case for its continued use in spermicidal preparations. However, reduced concentration of N9 in spermicidal preparations containing propranolol is an attractive option, as this would improve spermicidal efficacy, whilst retaining the protection against viral infection. It will also reduce the detergent-mediated side effects of N9.

Transmembrane ion-fluxes are critically important for sperm-motility initiation and regulation[25-27]. In particular, changes in intracellular calcium have been associated with sperm movement, hyper activation, acrosome reaction and fertilization[28-30]. To investigate whether the inhibition of sperm motility by treatment with propranolol is due to its ability to increase internal free Ca^{2+} concentration, the effects of this compound on sperm motility were tested both in presence and absence of extra cellular Ca^{+2}. Figures 9 (A and B) show the metabolic activity (lactate production) and motility of cells in calcium free and complete media.

A significant enhancement of the spermicidal action is observed in absence of Ca^{+2} as compared to that in complete medium. It is unclear why propranolol's efficiency in suppressing sperm metabolism/ movement should be increased in the absence of exogenous calcium. However, the existence of such an interaction raises the possibility of utilizing combinations of propranolol and chelating agents as novel and effective spermicidal agents.

CONCLUSION

Both racemic and the D-enantiomer of propranolol inhibit sperm motility in a dose dependent manner. Propronolol's spermicidal action depends upon its local anaesthetic properties rather than its β-blocking potential. The D-enantiomer, unlike the racemic DL is

devoid of the unwanted effects of ®-blockade and thus may be a useful drug. Propranolol and nonoxynol-9 interact in a synergistic manner to inhibit sperm motility. A significant enhancement of the spermicidal action of propranolol is observed in absence of Ca^{2+} over that in complete medium.

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