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Intrasperm Ca^{2+} modulation and human ejaculated sperm viability: influence of miconazole, clotrimazole and loperamide

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Abstract

Elevation of intrasperm Ca^{2+} is reported to influence viability of ejaculated spermatozoa. Human spermatozoa possess inositol triphosphate (IP_3)-sensitive Ca^{2+} stores, which can be targeted for increasing intrasperm Ca^{2+} level. The influence of agents affecting Ca^{2+} homeostasis has been investigated. Miconazole nitrate, clotrimazole and loperamide hydrochloride produced a dose- and time-dependent decrease in viability, each requiring respectively 0.5, 1.0 and 1.0 mM for producing death of all sperm cells immediately upon addition to ejaculated human semen samples. The reduction in sperm viability was accompanied by elevation of intrasperm Ca^{2+} and was not affected by presence or absence of extracellular Ca^{2+}. Significantly ($P < 0.05$) less time was required for producing complete loss of sperm viability and increasing intrasperm Ca^{2+} when any of these drugs was added to sperm cells previously treated with selected agents affecting Ca^{2+} homeostasis. This enhanced spermicidal activity of miconazole, clotrimazole and loperamide appeared to be due to further mobilization of Ca^{2+} from partially depleted intrasperm Ca^{2+} stores. Synergism of spermicidal activity and intrasperm Ca^{2+} elevation by miconazole or clotrimazole was observed when Ca^{2+} efflux from sperm cells was simultaneously inhibited by 2′,4′-dichlorobenzamil hydrochloride, a Na^+–Ca^{2+} exchange inhibitor. The spermicidal activity of miconazole, clotrimazole or loperamide due to elevation of intrasperm Ca^{2+} and its synergism, therefore, has great potential for exploitation of these drugs as contact spermicides.

Introduction

Intrasperm Ca^{2+} is known to play a vital role in sperm function and viability. Ca^{2+} homeostasis in sperm cells is intricately maintained through influx and efflux of Ca^{2+} via various channels and pumps present on sperm membrane. Pharmacological modulation of intrasperm Ca^{2+} concentration, [Ca^{2+}], has been investigated by treating human ejaculated semen samples with propranolol (White et al 1995), chelating agents (Lee et al 1996), 2′,4′-dichlorobenzamil hydrochloride (DBZ) (Reddy et al 2001; Patni et al 2001), membrane stabilizers (Moudgil et al 2002a, b), H_2-receptor antagonists (Gupta et al 2003) and H_1-receptor antagonists (Gupta et al 2004). These studies revealed that an elevation of intrasperm [Ca^{2+}] to ~1200 nM produced complete loss of viability of human ejaculated sperm cells. Hence, pharmacological modulation for achieving this critical intrasperm [Ca^{2+}] threshold seems to offer a feasible approach for contact spermicidal activity (Gupta et al 2005).

Imidazoles are known to inhibit store operated calcium (SOC) channels. However, miconazole has been observed to elevate intracellular Ca^{2+} in thymic lymphocytes (Mason et al 1993) and HL-60 cells (Harper et al 1997; Harper & Daly 1999). Similarly, clotrimazole has been reported to elevate intracellular Ca^{2+} in HL-60 cells (Harper et al 1997; Harper & Daly 1999). These effects of miconazole and clotrimazole were suggested to be due to calcium ATPase

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inhibition (Mason et al 1993; Snajdrova et al 1998; Lax et al 2002). In addition, miconazole and clotrimazole have been reported to increase phosphoinositide breakdown (Daly et al 1995), suggesting involvement of the inositol triphosphate (IP_3) pathway in the release of intracellular Ca^{2+}. Loperamide augments Ca^{2+} influx through activated SOC channels (Harper et al 1997). Furthermore, elevation of intracellular Ca^{2+} level in insulin-secreting HIT-T15 cells following treatment with loperamide was reported to be due to its effect on IP_3-gated intracellular Ca^{2+} stores (He et al 2003).

The presence of IP_3 receptors on the neck and acrosome of human spermatozoa (Naaby-Hansen et al 2001) and an elevation of intrasperm [Ca^{2+}] following treatment of human ejaculated sperm with Ca-ATPase inhibitors (Gupta et al 2003; Williams & Ford 2003) or IP_3 modulators (Gupta et al 2004) led to the hypothesis that miconazole, clotrimazole or loperamide could possess the ability of modulating intrasperm Ca^{2+} level in human ejaculated sperm cells.

Therefore, in this study we have evaluated the influence of miconazole, clotrimazole and loperamide on [Ca^{2+}] and the viability of sperm in human ejaculated semen samples.

Materials and Methods

Miconazole and clotrimazole were gift samples from Micro Labs Limited (Tamil Nadu, India) and Cipla Pharmaceuticals Limited (Mumbai, India), respectively. Loperamide was obtained as a gift sample from Torrent Pharmaceuticals Limited (Gandhinagar, India). DBZ was obtained as a gift sample from SRI International (USA). Histamine dihydrochloride was purchased from Hi Media Laboratory Limited (Mumbai, India). Quin 2-AM was purchased from Sigma Chemicals (USA). All other chemicals used in this study were purchased from Qualigens (Mumbai, India) and were of AR grade.

Preparation of drug solutions

Absolute ethanol, acetone or a 1:1 mixture of methanol and dimethylsulfoxide was used in a concentration of 1% (v/v) in Biggers–Whitten–Whittingham (BWW) medium for preparing stock solutions of loperamide, clotrimazole or miconazole, respectively. Further dilutions of these drugs were made with BWW medium. Solutions of DBZ or histamine dihydrochloride were prepared in BWW medium. Quin 2-AM was dissolved in dimethylsulfoxide. Ca^{2+}-free BWW medium was prepared by substituting calcium chloride with an equimolar concentration of sodium chloride in the normal BWW medium along with the addition of EDTA (100 μM).

Semen collection

Semen samples exhibiting $> 20 \times 10^6$ spermatozoa mL^{-1}, $> 60\%$ motility and $> 60\%$ normal morphology (WHO manual 1999) were collected from human volunteers (22–25 years old) by masturbation into a warm, sterile glass beaker. Freshly ejaculated samples from six volunteers (non-smokers and non-alcoholics), after an abstinence from sexual activity of not less than 48 h but not more than five days, were used for all experiments. Each experiment was carried out on a semen sample from each volunteer (crossover design).

Sperm motility analysis

The effect of miconazole, clotrimazole, loperamide, histamine dihydrochloride or DBZ alone on sperm motility was studied by mixing their respective solutions with an equal volume of liquefied semen.

Experiments aimed at studying the effect of combinations of DBZ with miconazole or clotrimazole on sperm motility employed the addition of the two drug solutions and semen in the ratio of 0.5:0.5:1.0. Other drug combinations involved the addition of a second drug solution after exposing the sperm cells to the first drug solution for different time intervals.

The respective drug–semen admixtures were incubated at $37 \pm 2^{\circ}C$. A 0.1-mL sample was removed at different time intervals, gently mixed with 0.05 mL eosin (Y) dye solution (0.5% w/v in normal saline), and examined for dead (stained red) and alive (unstained) spermatozoa. Not less than 200 sperm cells were counted and the results expressed as fractional motility. Fractional motility (FM) was calculated by employing the formula, FM = % motile sperm in drug-treated semen sample/% motile sperm in control semen sample.

Sperm revival test

Glucose solution was added to a sample of totally immotile sperm (final concentration of glucose adjusted to 250 mg mL^{-1}) and the mixture incubated for 60 min at $37 \pm 2^{\circ}C$ (Reddy et al 1996). After incubation, the specimen was examined for revival of sperm motility.

Measurement of intrasperm [Ca^{2+}]

Intrasperm [Ca^{2+}] was monitored in various drug–semen admixtures used for sperm motility analysis. Intrasperm [Ca^{2+}] was determined by measuring the fluorescence signal emitted by the Ca^{2+} chelating agent, Quin 2-AM, using a Perkin Elmer LS 50B spectrofluorimeter. The excitation and emission wavelengths employed were 339 nm and 492 nm, respectively. Intrasperm [Ca^{2+}] was calculated according to the method reported by White et al (1995).

Data analysis

The results are expressed as mean $\pm$ s.d. of experiments conducted on semen samples of six volunteers.

Comparison between mean values of different groups for statistical significance was done by employing analysis of variance. This was followed by Studentized Range test for comparing statistical significance between two groups. The level of significance was fixed at $P < 0.05$.

Results and Discussion

Effect of miconazole, clotrimazole or loperamide alone on sperm motility

Miconazole (Figure 1A), clotrimazole (Figure 1B) and loperamide (Figure 1C) produced a dose- and time-dependent decrease in sperm motility. A dose of 0.5 mM miconazole or 1.0 mM clotrimazole or loperamide produced a complete loss of sperm viability immediately upon addition to human ejaculated semen samples. The dead sperm cells could not be revived even after incubation in glucose solution, indicating irreversible loss of sperm cell viability by all these drugs. Further, removal of extracellular Ca^{2+} did not significantly alter the effect of these drugs on sperm motility. Hence, Ca^{2+} influx from extracellular source did not appear to play a major role in the spermicidal action of these drugs.

Effect of miconazole, clotrimazole or loperamide on intrasperm [Ca^{2+}]

It was evident from Figure 1A–C that the intrasperm [Ca^{2+}] remained almost constant after 30–35 min due to death of all the sperm cells. Therefore, the spermicidal action by these drugs could be ascribed to elevation of intrasperm [Ca^{2+}] to the threshold level of 1100–1200 nM. This finding was in agreement with earlier reports (White et al 1995; Lee et al 1996; Patni et al 2001; Reddy et al 2001; Gupta et al 2003, 2004).

Miconazole (20 μM) has been reported to elevate intracellular Ca^{2+} in thymic lymphocytes by approximately sixfold. This effect lasted for only 10 min (Mason et al 1993). Addition of miconazole (2 μM) to HL-60 cells caused a single fold (Harper et al 1997) or a 0.3-fold increase in intracellular Ca^{2+} over the basal level that lasted for approximately 5 min (Harper & Daly 1999). Similarly, addition of clotrimazole (10 μM) to HL-60 cells was reported to transiently elevate intrasperm Ca^{2+} by 0.75-fold for approximately 5 min (Harper et al 1997; Harper & Daly 1999). These observations were recorded in the presence of extracellular Ca^{2+}. Harper & Daly (1999) reported that miconazole or clotrimazole alone both produced concentration-dependent increases in intracellular Ca^{2+} level in HL-60 cells after incubation in Ca^{2+}-free media. However, the authors did not compare the elevation of intracellular [Ca^{2+}] observed in the presence or absence of extracellular Ca^{2+}. The observed insignificant ($P > 0.05$) elevation of intrasperm [Ca^{2+}] accompanied with the insignificant ($P > 0.05$) loss of motility (Figure 1A, B) by miconazole (0.025 mM) or clotrimazole (0.25 mM) in normal BWW as compared with that in Ca^{2+}-free BWW medium strongly suggested a negligible role of Ca^{2+} influx in the spermicidal activity of both drugs.

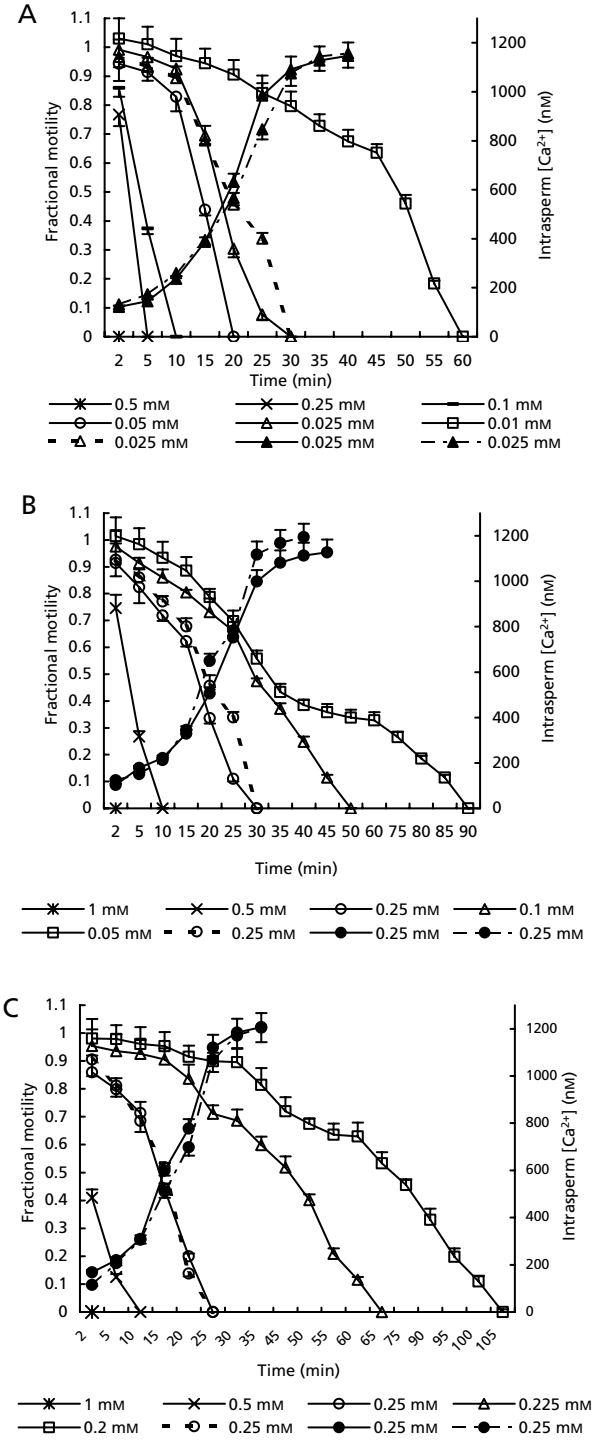

Figure 1 Effect of miconazole (A), clotrimazole (B) or loperamide (C) alone on motility of sperm (blank symbols) and intrasperm [Ca^{2+}] (filled symbols) in ejaculated human semen samples in the presence (solid lines) or absence (broken lines) of extracellular calcium.

Loperamide (30 μM) has been reported not to elevate intracellular Ca^{2+} in NIH3T3, DDT-MF2, RBL-2H3 and GH_4C_1 cells. However, it increased the intracellular Ca^{2+} level by 0.5-fold in astrocytoma 1321N cells. This effect was transient and lasted for only 4 min (Harper et al 1997). In another study involving insulin secreting HIT-T15 cells, loperamide (0.25 mM) was observed to produce a dose-dependent increase in intracellular Ca^{2+}. This effect was found to persist even in the absence of extracellular Ca^{2+} (He et al 2003). Figure 1C shows that the elevation of intrasperm Ca^{2+} by loperamide (0.25 mM) in normal BWW medium was not significantly different ($P > 0.05$) compared with that in the absence of extracellular Ca^{2+}.

Drug combinations: potentiation of spermicidal activity and intrasperm [Ca^{2+}]

DBZ (2 mM) and histamine dihydrochloride (75 mM) have been reported to produce complete loss of sperm viability accompanied with achievement of critical threshold [Ca^{2+}] in ejaculated human sperm cells, respectively, in 60 min and 50 min (Patni et al 2001; Gupta et al 2004). Miconazole (0.025 mM) or clotrimazole (0.25 mM) alone produced complete loss of sperm viability in 30 min (Figure 1A, B). However, addition of miconazole (0.025 mM) or clotrimazole (0.25 mM) to sperm pretreated with histamine dihydrochloride (75 mM) for different time periods was observed to produce complete loss of sperm viability as well as achieve the critical threshold intrasperm [Ca^{2+}] in significantly ($P < 0.05$) less duration as compared with the effect of histamine dihydrochloride alone. In addition, these effects were not significantly ($P > 0.05$) different in Ca^{2+}-free BWW medium (Table 1). The observed elevation of intrasperm [Ca^{2+}] in Ca^{2+}-free medium was in agreement with an earlier report where histamine dihydrochloride-induced intracellular [Ca^{2+}] elevation in human glomerular epithelial cells persisted even in the absence of extracellular [Ca^{2+}] (Spath et al 1994). The preliminary incubation with histamine dihydrochloride could be expected to release Ca^{2+} through activation of IP_3 receptors. However, subsequent addition of miconazole or clotrimazole might have resulted in activation of IP_3 receptors (Daly et al 1995) or may have inhibited the endoplasmic reticulum Ca^{2+}-ATPase (Snajdrova et al 1998; Lax et al 2002). Both these mechanisms are known to elicit an elevation of cytoplasmic [Ca^{2+}]. Similarly, addition of loperamide to sperm pretreated with miconazole (0.025 mM) or clotrimazole (0.25 mM) for different time periods produced complete loss of viability in significantly ($P < 0.05$) less duration as compared with their effect when administered alone, irrespective of the presence or absence of extracellular Ca^{2+} (Table 2). Therefore, the observed augmentation of intrasperm [Ca^{2+}] indicated the capability of each drug under study (miconazole, clotrimazole or loperamide) to further mobilize intrasperm Ca^{2+} from partially depleted Ca^{2+} stores.

The Na^+–Ca^{2+} exchanger present in sperm cells plays a pivotal role in extruding cytoplasmic Ca^{2+} (Patni et al 2001). The time required to produce a complete loss of sperm viability (10 min) by using a combination of DBZ and miconazole or clotrimazole (Figure 2) was significantly ($P < 0.05$) lower as compared with that required by miconazole (30 min), clotrimazole (30 min) or DBZ (60 min) alone. This marked synergism in spermicidal action could be ascribed to elevation of intrasperm [Ca^{2+}] by miconazole or clotrimazole coupled with prevention of its efflux due to inhibition of Na^+–Ca^{2+} exchanger by DBZ.

Conclusion

The addition of miconazole, clotrimazole or loperamide alone to human ejaculated semen samples produced a marked increase (11–12-fold over basal level) in intrasperm [Ca^{2+}], which remained unaffected in the absence of extracellular Ca^{2+}. This effect accompanied a decrease in sperm viability. The ability of these drugs to influence IP_3-sensitive Ca^{2+} stores (Harper & Daly 1999; He et al 2003) and inhibit endoplasmic reticulum Ca^{2+}-ATPase (Snajdrova et al 1998; Lax et al 2002) could be responsible for elevating intrasperm [Ca^{2+}]. The results of drug combination studies suggested the ability of miconazole, clotrimazole or loperamide to further mobilize Ca^{2+} from partially depleted intrasperm Ca^{2+} stores. In addition, DBZ was found to synergize the spermicidal action of miconazole and clotrimazole.

Overall, the findings suggested a new use of these approved drugs that are already being clinically used for treating candidal vulvo-vaginitis (miconazole and clotrimazole) and acute diarrhoea (loperamide). The concentration of miconazole (0.01 mM) that is reported to be effective against *Candida albicans* (Kucers & Bennett 1979) was found to produce complete loss of human ejaculated sperm viability in 60 min. However, clotrimazole required a higher concentration of 0.1 mM to produce the same effect, suggesting it to be ten-times less potent as a spermicide than miconazole. Similar observations regarding potency of clotrimazole and miconazole with respect to calcium increase in HL-60 cells have been reported by Harper & Daly (1999). Nevertheless, miconazole and clotrimazole both require long-term intravaginal application for clinical effectiveness, and so their spermicidal potential needs to be considered critically to rule out the possibility of unexplained contraception. Furthermore, observed synergism of spermicidal activity could be exploited for formulating an efficacious and safe contact spermicidal formulation of miconazole or clotrimazole for contraception.

Table 1 The effect of miconazole (0.025 mM) or clotrimazole (0.25 mM) on motility and intrasperm [Ca^{2+}] upon addition to sperm cells after pretreatment with histamine dihydrochloride (75 mM) for different time intervals

Treatment	Time (min)	Miconazole (0.025 mM) added to histamine dihydrochloride (75 mM) pretreated sperm cells after							
		5 min		15 min		25 min		35 min	
		FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM
Normal BWW	2	0.95 ± 0.05	138.29 ± 6.72	0.89 ± 0.03	118.37 ± 6.12	0.93 ± 0.06	147.65 ± 7.53	0.97 ± 0.05	109.67 ± 5.68
	10	0.19 ± 0.01	728.95 ± 37.14	0.53 ± 0.04	216.74 ± 9.78	0.65 ± 0.01	237.58 ± 12.03	0.67 ± 0.02	248.59 ± 12.84
	20	0	1211.91 ± 60.87	0.11 ± 0.01	856.14 ± 43.24	0.45 ± 0.02	412.08 ± 21.13	0.45 ± 0.03	462.10 ± 23.47
	30		1221.58 ± 62.13	0	1193.04 ± 59.81	0	1078.19 ± 54.24	0.31 ± 0.01	789.29 ± 40.11
	40						1217.54 ± 61.02	0	1208.65 ± 61.23
Ca^{2+}-free BWW	2	0.92 ± 0.06	127.56 ± 6.41	0.96 ± 0.05	135.89 ± 7.11	0.88 ± 0.06	127.43 ± 6.74	0.94 ± 0.05	117.32 ± 6.13
	10	0.14 ± 0.01	746.34 ± 37.92	0.58 ± 0.04	289.58 ± 13.97	0.62 ± 0.04	274.36 ± 13.34	0.70 ± 0.04	284.51 ± 14.86
	20	0	1156.23 ± 58.37	0.13 ± 0.01	871.08 ± 43.48	0.44 ± 0.02	446.82 ± 22.44	0.52 ± 0.03	485.02 ± 23.89
	30			0	1178.42 ± 58.55	0	1141.05 ± 57.31	0.31 ± 0.03	810.33 ± 40.21
	40				1210.34 ± 60.07		1174.58 ± 57.59	0	1196.23 ± 59.54
Treatment	**Time (min)**	**Clotrimazole (0.25 mM) added to histamine dihydrochloride (75 mM) pretreated sperm cells after**							
		5 min		15 min		25 min		35 min	
		FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM
Normal BWW	2	0.95 ± 0.05	119.99 ± 6.05	0.92 ± 0.06	128.34 ± 6.62	0.86 ± 0.05	135.24 ± 6.91	0.93 ± 0.06	109.36 ± 5.61
	10	0.38 ± 0.02	522.61 ± 26.36	0.62 ± 0.03	314.28 ± 16.03	0.58 ± 0.04	312.39 ± 15.85	0.71 ± 0.02	267.49 ± 12.9
	20	0	1018.29 ± 51.24	0.17 ± 0.01	794.87 ± 39.86	0.37 ± 0.02	462.06 ± 23.41	0.51 ± 0.03	476.14 ± 23.55
	30		1159.18 ± 57.74	0	1162.39 ± 57.49	0	1087.39 ± 53.73	0.32 ± 0.01	791.22 ± 39.36
	40						1187.33 ± 59.24	0	1205.30 ± 60.30
Ca^{2+}-free BWW	2	0.87 ± 0.06	127.58 ± 6.40	0.96 ± 0.06	109.66 ± 5.62	0.93 ± 0.06	152.31 ± 7.34	0.89 ± 0.06	114.25 ± 5.46
	10	0.35 ± 0.02	574.12 ± 28.45	0.74 ± 0.05	263.75 ± 12.76	0.79 ± 0.05	305.81 ± 15.46	0.61 ± 0.02	350.49 ± 17.43
	20	0	1115.76 ± 55.23	0.17 ± 0.01	814.10 ± 40.88	0.47 ± 0.04	488.65 ± 24.22	0.45 ± 0.01	510.42 ± 25.17
	30		1206.59 ± 59.95	0	1152.78 ± 57.59	0	1085.26 ± 53.79	0.13 ± 0.01	858.16 ± 41.74
	40						1202.44 ± 59.89	0	1185.23 ± 58.86

FM, fractional motility. Blank rows indicate termination of experiment after observing 100% immotility. Values of FM and [Ca^{2+}] are expressed as mean ± s.d. of six volunteers.

Table 2 The effect of loperamide (0.25 mM) on motility and intrasperm [Ca^{2+}] upon addition to sperm cells after pretreatment with miconazole (0.025 mM) or clotrimazole (0.25 mM) for different time intervals

Treatment	Time (min)	Loperamide (0.25 mM) added to miconazole (0.025 mM) pretreated sperm cells after					
		5 min		10 min		15 min	
		FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM
Normal BWW	2	0.95 ± 0.06	113.38 ± 8.51	0.90 ± 0.06	137.53 ± 8.41	0.91 ± 0.06	125.36 ± 8.47
	10	0.18 ± 0.01	571.85 ± 26.44	0.26 ± 0.03	673.15 ± 31.24	0.31 ± 0.02	685.72 ± 32.57
	20	0	1152.20 ± 58.10	0	1189.76 ± 58.87	0	1175.06 ± 57.80
	25		1217.19 ± 61.95		1203.55 ± 60.72		1198.61 ± 59.43
Ca^{2+}-free BWW	2	0.94 ± 0.05	122.81 ± 8.59	0.96 ± 0.06	119.35 ± 7.81	0.90 ± 0.03	131.24 ± 7.76
	10	0.22 ± 0.02	740.6 ± 35.06	0.37 ± 0.03	614.29 ± 30.25	0.33 ± 0.03	658.40 ± 31.87
	20	0	1201.66 ± 59.63	0	1159.19 ± 55.43	0	1154.03 ± 57.48
	25		1267.35 ± 62.76		1186.27 ± 60.44		1186.25 ± 58.79
Treatment	**Time (min)**	**Loperamide (0.25 mM) added to clotrimazole (0.25 mM) pretreated sperm cells after**					
		5 min		10 min		15 min	
		FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM	FM	[Ca^{2+}], nM
Normal BWW	2	0.84 ± 0.06	138.29 ± 8.42	0.92 ± 0.07	124.65 ± 9.84	0.97 ± 0.06	108.27 ± 8.87
	10	0.39 ± 0.03	586.10 ± 28.16	0.30 ± 0.02	593.04 ± 29.12	0.35 ± 0.03	674.16 ± 31.06
	20	0	1073.24 ± 51.09	0	1124.36 ± 55.81	0	1046.28 ± 51.78
	25		1118.17 ± 56.78		1184.23 ± 58.64		1104.62 ± 54.23
Ca^{2+}-free BWW	2	0.96 ± 0.06	109.87 ± 5.63	0.95 ± 0.03	117.15 ± 4.76	0.91 ± 0.07	141.26 ± 8.43
	10	0.41 ± 0.04	603.83 ± 31.57	0.29 ± 0.03	614.26 ± 31.24	0.27 ± 0.03	703.44 ± 34.27
	20	0	1167.5 ± 58.87	0	1114.27 ± 55.45	0	1143.62 ± 56.55
	25		1227.97 ± 60.89		1152.63 ± 57.91		1182.29 ± 59.63

FM, fractional motility. Blank rows indicate termination of experiment after observing 100% immotility. Values of FM and [Ca^{2+}] are expressed as mean ± s.d. of six volunteers.

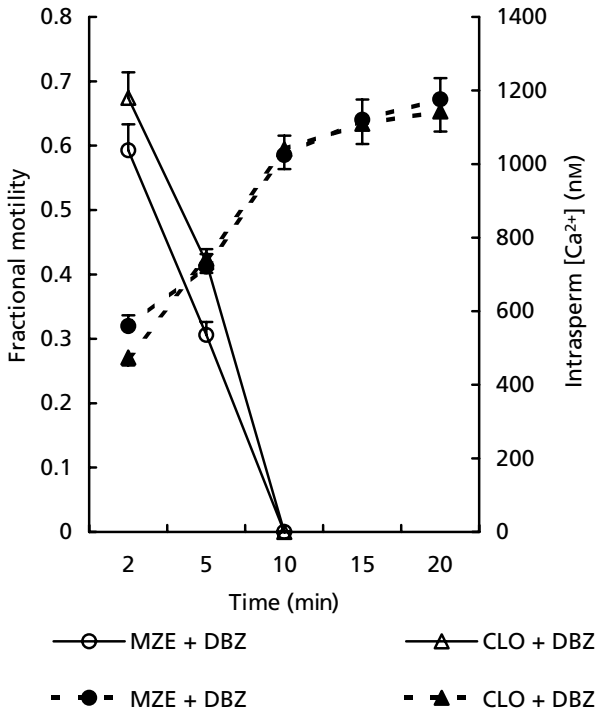

Figure 2 Influence of solutions containing admixtures of 2′,4′-dichlorobenzamil hydrochloride (DBZ, 2 mM) and miconazole (MZE, 0.025 mM) or clotrimazole (CLO, 0.25 mM) on sperm motility (solid lines) and intrasperm Ca^{2+} (broken lines) in human ejaculated semen samples.

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