JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1387–1392

Received November 17, 2000
Accepted June 21, 2001
ISSN 0022-3573

Role of intracellular calcium in the spermicidal action of 2′,4′-dichlorobenzamil, a novel contact spermicide

Anil K. Patni, Sunil Gupta, Ajay Sharma, Ashok K. Tiwary and Santosh K. Garg

Abstract

The Na^+–Ca^{2+} exchanger and Ca^{2+}-ATPase pumps reported to be present on the sperm membrane are responsible for maintaining the intracellular Ca^{2+} concentration that is involved in regulation of sperm function. We have investigated the role of intracellular Ca^{2+} in the presence of 2′,4′-dichlorobenzamil hydrochloride (benzamil), a Na^+–Ca^{2+} exchange inhibitor, on human sperm motility. The mechanism of the complementary spermicidal action produced by a combination of benzamil and propranolol on human spermatozoa has been investigated also. When administered alone benzamil and propranolol produced a dose- and time-dependent decrease in motility of sperm in ejaculated semen and spermatozoa separated from semen. A combination of benzamil and propranolol exhibited a complementary spermicidal action, thereby resulting in dose reduction of both drugs for obtaining total immotility within 1 min of administration. An increase in the intracellular Ca^{2+} level was found to contribute to the spermicidal activity. Inhibition of the Na^+–Ca^{2+} exchange system on sperm membrane by benzamil and membrane stabilization by propranolol resulted in accumulation of Ca^{2+} inside the sperm cells. When the two drugs were used in combination the time required for the total loss of motility of spermatozoa was significantly reduced due to a similar mechanism of action of both drugs.

Department of Pharmaceutical Sciences & Drug Research, Punjabi University, Patiala 147 002, India

Anil K. Patni, Sunil Gupta, Ajay Sharma, Ashok K. Tiwary

Department of Pharmacology, Post Graduate Institute of Medical Education & Research, Chandigarh, India

Santosh K. Garg

Correspondence: A. K. Tiwary, Department of Pharmaceutical Sciences & Drug Research, Punjabi University, Patiala 147 002, India.
E-mail: tiwary@pbi.ernet.in; akiwary@rediffmail.com

Acknowledgements: The authors gratefully acknowledge the help of SRI International, USA, for providing a gift sample of 2′,4′-dichlorobenzamil hydrochloride as part of the Chemical Synthesis Program of the National Institute of Mental Health, contract N01 MH30003.

Introduction

The sperm membrane is reported to possess a Na^+–Ca^{2+} exchanger (Bradley & Forrester 1980) and a Ca^{2+}-ATPase pump (Breitbart et al 1984). Both these systems play a vital role in extrusion of Ca^{2+} from the sperm cell (Ashraf et al 1982).

One of the causes of loss of sperm motility is an increase in the intracellular Ca^{2+} level (White et al 1995). 2′,4′-Dichlorobenzamil hydrochloride (benzamil) has been reported to possess Na^+–Ca^{2+} exchange inhibitory activity (Siegel et al 1984; Deshpande et al 1997), as well as Mg^{2+}-dependent Ca^{2+}-ATPase inhibitory activity (Siegel et al 1984) and has been demonstrated to increase the intracellular Ca^{2+} level in cardiac cells. Hence, it is envisaged that benzamil may exhibit spermicidal activity due to the elevation of intracellular Ca^{2+}.

Propranolol has been reported to produce sperm death due to an increase in intracellular Ca^{2+}. This action may be due to its membrane stabilizing property and not related to its β-blocking property (White et al 1995). Therefore, a combination of benzamil and propranolol would be expected to exhibit a synergistic spermicidal action.

This study was designed to investigate the mechanism of spermicidal action of benzamil and propranolol on ejaculated human sperm. Furthermore, various combinations of both drugs were tested to establish the mechanism of complementary spermicidal action that would help in reducing the dose of the respective drugs.

Materials and Methods

2′,4′-Dichlorobenzamil hydrochloride (benzamil) was a gift sample from SRI International USA. Propranolol I. P. was a gift sample from Crystal Pharmaceuticals, Ambala, India. Quin 2-AM was purchased from Sigma Chemicals, USA. Solutions of benzamil and propranolol were prepared in Briggers-Whitten-Whittingham (BWW) medium. Quin 2-AM was dissolved in dimethyl sulfoxide. All other chemicals used in this study were purchased from Qualigens, Mumbai, India, and were of AR grade.

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 of not less than 48 h but not more than five days (Reynolds & Narang 1984), were used for all experiments. Each experiment was carried out on a semen sample from each volunteer (crossover design).

Separation of spermatozoa from semen

Spermatozoa were separated from semen by centrifuging the liquefied semen in BWW medium (1:1) at 1000 rev min^{-1} for 10 min. The pellet obtained after decanting the supernatant was suspended in BWW medium and again centrifuged at 1000 rev min^{-1} for 10 min. The pellet obtained was finally suspended in BWW medium so as to yield $> 20 \times 10^6$ spermatozoa mL^{-1}.

Sperm motility analysis

Liquefied semen or spermatozoa suspended in BWW medium were mixed with a solution of benzamil, propranolol or a combination of both drugs (1:1) and incubated at 37 ± 2°C. A 0.1-mL sample was taken out at different time intervals, gently mixed with eosin (Y) dye solution (0.05 mL) and examined for dead (stained red) and alive (unstained) sperm. Not less than 250 sperm were counted and the results expressed as 'fractional motility' (% motile sperm in presence of drug/% motile sperm in control) at each time interval.

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 ± 2°C (Reddy et al 1996). After incubation, the specimen was examined for revival of sperm motility.

Measurement of intracellular Ca^{2+} in spermatozoa

The influence of benzamil, propranolol or drug combination on the intracellular Ca^{2+} was assessed in spermatozoa separated from semen by measuring the fluorescence signal emitted by the calcium chelating agent Quin 2-AM, according to the method outlined by White et al (1995).

Data analysis

Results are expressed as mean ± s.d. of experiments conducted on samples from six volunteers. Statistical analysis was performed by paired t-test. $P < 0.05$ was considered to be statistically significant.

Results

Effect of benzamil on sperm motility

Benzamil produced a decrease in sperm motility at all dose levels (0.25–2.0 mM). A dose of 4.0 mM produced complete immotility immediately upon addition to semen samples (Figure 1A). The spermicidal efficacy of benzamil was apparently more in spermatozoa samples as compared with total semen (Figure 1B).

Effect of propranolol on sperm motility

Propranolol 6.0 mM produced complete immotility immediately upon addition to semen samples (Figure 2A). The same effect was observed with 1.2 mM propranolol in spermatozoa separated from semen (Figure 2B).

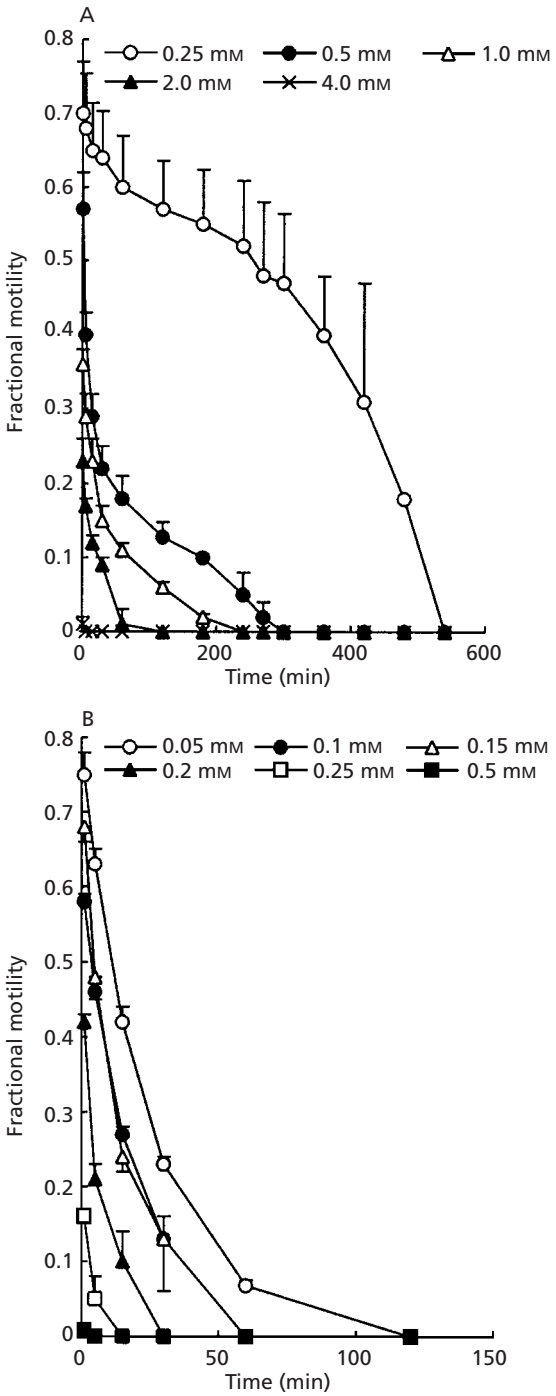

Figure 1 Effect of benzamil on motility of (A) sperm in human semen samples and (B) spermatozoa separated from human semen.

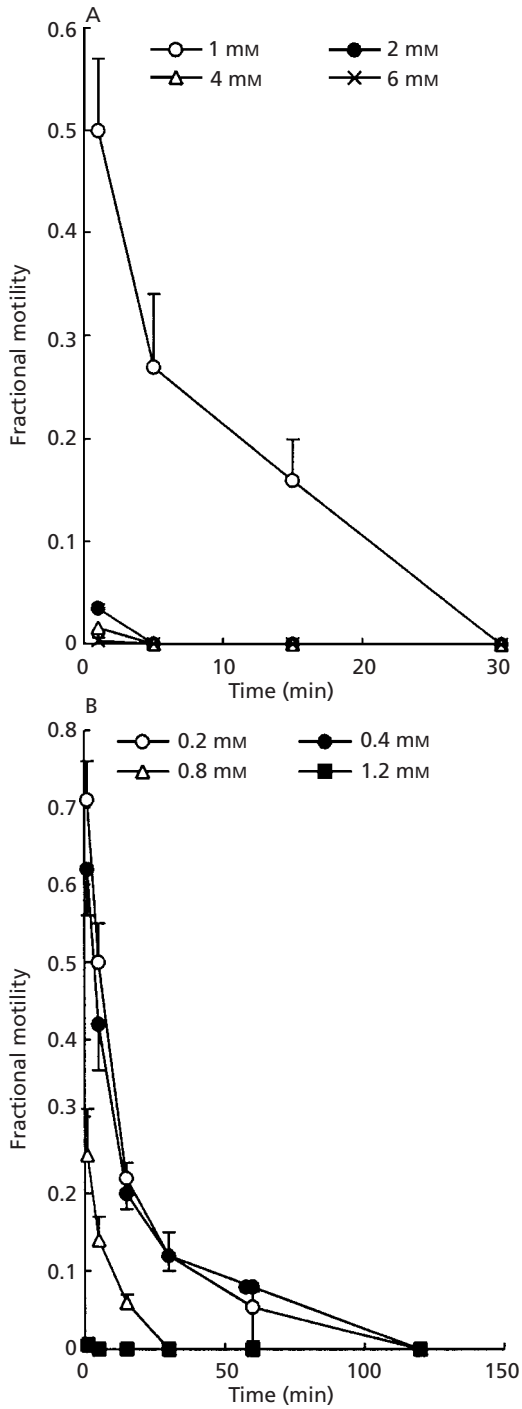

Figure 2 Effect of propranolol on motility of (A) sperm in human semen samples and (B) spermatozoa separated from human semen.

Effect of benzamil–propranolol combination on sperm motility

Propranolol (1.0 mM) enhanced the spermicidal activity of benzamil (0.5, 1.0 and 2.0 mM) in semen samples. Similarly, benzamil (1.0 mM) enhanced the spermicidal activity of propranolol (1.0 or 2.0 mM) in semen samples (Table 1).

Table 1 Spermicidal activity of 2′,4′-dichlorobenzamil hydrochloride (benzamil), propranolol and their combination in human semen samples.

	Benzamil only (mM)			Propranolol (1 mM) + benzamil* (mM)			Propranolol only (mM)		Benzamil (1 mM) + propranolol# (mM)	
	0.5	1.0	2.0	*0.5	*1.0	*2.0	1.0	2.0	#1.0	#2.0
100% immotility (min)	288 ± 16	171 ± 20	60 ± 10	26 ± 4	12 ± 4	8 ± 2	28 ± 2	4.2 ± 1	12 ± 4	1.4 ± 0.5

Time for 100% immotility (min) indicates mean value ± s.d. of six volunteers.

Table 2 Spermicidal activity of 2′,4′-dichlorobenzamil hydrochloride (benzamil), propranolol and their combination in samples containing spermatozoa separated from human semen.

	Benzamil only (mM)			Propranolol (0.2 mM) + benzamil* (mM)			Propranolol only (mM)			Benzamil (0.2 mM) + propranolol# (mM)		
	0.05	0.1	0.2	*0.05	*0.1	*0.2	0.2	0.4	0.8	#0.2	#0.4	#0.8
100% immotility (min)	111 ± 13	51 ± 8	25 ± 5	23 ± 5	13 ± 2	6.2 ± 3	102 ± 12	99 ± 13	24 ± 4	6.2 ± 3	3.4 ± 2	1.4 ± 0.5

Time for 100% immotility (min) indicates mean value ± s.d. of six volunteers.

In samples containing spermatozoa separated from semen, propranolol (0.20 mM) enhanced the spermicidal activity of benzamil. Similarly, in the presence of 0.20 mM benzamil, an enhancement of spermicidal activity by propranolol (0.20, 0.40 and 0.80 mM) in spermatozoal samples was observed (Table 2).

Effect of drug treatment on intracellular Ca^{2+}

The intracellular Ca^{2+} increased slowly with time when either benzamil (0.05 mM) or propranolol (0.20 mM) was used alone and total loss of motility was observed at 111 ± 13 min and 102 ± 12 min, respectively. The time for total immotility was reduced to 23 ± 5 min by using a combination of both drugs (Figure 3).

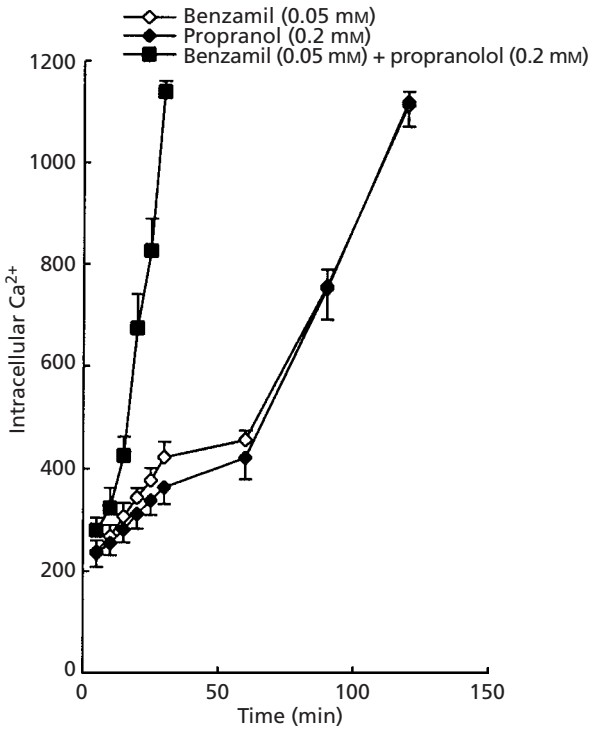

Figure 3 Effect of 0.05 mM benzamil, 0.2 mM propranolol or a combination of 0.05 mM benzamil and 0.2 mM propranolol on intracellular calcium level in spermatozoa separated from human semen.

Discussion

A dose of 0.50 mM benzamil was required to produce total immotility immediately upon addition to spermatozoa separated from semen compared with a dose of 4.0 mM for semen samples (Figure 1A and B). This indicated a significant 8-fold increase in the efficacy of benzamil on spermatozoa separated from semen. A similar finding was reported for magainins (Edelstein et

al 1991; Reddy et al 1996). Although no exact reason for the findings can be suggested, the role of certain substances in semen that bind with benzamil and the high viscosity of seminal fluid that restricts intimate contact of benzamil with sperm cannot be ruled out. Nonoxynol-9, the currently widely used spermicide has been reported to produce complete immotility of spermatozoa within 1 min of addition at a concentration of 0.81 mM (White et al 1995). Hence, the data showed benzamil to be 1.62-fold more potent than nonoxynol-9.

Motility studies showed a significant 5-fold-reduction in the dose of propranolol to produce 100% immotility immediately after addition to spermatozoa separated from semen as compared with semen samples (Figure 2A and B). It is worth noting that the total loss of motility in seminal samples after the addition of 1 mM propranolol had occurred by 30 min (Figure 2A). In the samples containing spermatozoa separated from semen, total loss of motility was observed 30 min after addition of 0.8 mM propranolol (Figure 2B). Therefore, the spermicidal activity of propranolol does not seem to be a linear function of the dose.

Benzamil (0.50, 1.0 and 2.0 mM) alone produced complete immotility in semen samples at 288 ± 16, 171 ± 20 and 60 ± 10 min, respectively. However, in the presence of propranolol (1.0 mM), complete immotility was observed at 26 ± 4, 12 ± 4 and 8 ± 2 min, respectively (Table 1). This indicated a significant enhancement of the spermicidal activity of benzamil by propranolol in sperm samples. On the other hand, benzamil below a concentration of 1.0 mM was found to be ineffective in significantly enhancing the spermicidal efficacy of propranolol (1.0 or 2.0 mM) in semen samples. This may be due to partial neutralization of benzamil by certain substances present in seminal fluid that reduced its efficacy in semen. Benzamil was effective at a concentration of 1.0 mM in enhancing the spermicidal activity of propranolol (Table 1).

Benzamil (0.05, 0.10 and 0.20 mM) alone produced complete immotility at 111 ± 13, 51 ± 8 and 25 ± 5 min, respectively, in samples containing spermatozoa separated from semen. In the presence of propranolol (0.20 mM), the same effect was observed at 23 ± 5, 13 ± 2 and 6.2 ± 3 min, respectively (Table 2). This indicated a significant enhancement of spermicidal efficacy of benzamil by propranolol. Similarly, the time required for complete immotility by propranolol (0.20, 0.40 and 0.80) was 102 ± 12, 99 ± 13 and 24 ± 4 min, respectively. The addition of 0.20 mM benzamil significantly reduced the time required by propranolol to exhibit complete immotility to 6.2 ± 3, 3.4 ± 2 and 1.4 ± 0.5 min, respectively (Table 2).

It is interesting to note that 1.0 mM benzamil and 2.0 mM propranolol alone produced complete immotility in semen samples at 171 ± 20 and 4.2 ± 1 min, respectively. However, in combination, complete immotility had occurred at 1.4 min. Hence, benzamil enhanced the spermicidal activity of propranolol by approximately 3-fold in semen samples. In samples containing spermatozoa separated from semen, 0.20 mM benzamil and 0.80 mM propranolol alone required 25 ± 5 and 24 ± 4 min to produce complete immotility, respectively. A combination of both drugs produced complete immotility at 1.4 min. This showed that benzamil enhanced the spermicidal activity of propranolol in samples of spermatozoa by 18-fold. These findings suggested that benzamil was 6-times more effective in enhancing the spermicidal activity of propranolol in spermatozoa separated from semen as compared with that in total semen samples.

Ionophores (Hong et al 1986) and propranolol (White et al 1995) are known to produce spermicidal action by increasing the intracellular Ca^{2+}. A combination of 0.05 mM benzamil and 0.2 mM propranolol was selected for intracellular Ca^{2+} study because it produced total loss of motility at 23 min and, hence, allowed time-dependent measurement of intracellular Ca^{2+}. It is evident from Figure 3 that the intracellular Ca^{2+} rose slowly with time when either 0.05 mM benzamil or 0.20 mM propranolol was used alone. However, a combination of both drugs produced a rapid increase in intracellular Ca^{2+}, resulting in a significant decrease in the time required for total immotility. This seemed to be due to the complementary action of benzamil and propranolol. Benzamil prevented Ca^{2+} efflux from spermatozoa due to the inhibition of the Na^{+}–Ca^{2+} exchange system and Mg^{2+}-dependent Ca^{2+}-ATPase activity, similar to that observed in cardiac cells (Siegel et al 1984; Deshpande et al 1997). Propranolol perhaps altered the sperm membrane structure due to its membrane stabilizing action that may have in turn influenced the Ca^{2+} efflux mechanism. Therefore, both drugs acted synergistically to elevate intracellular Ca^{2+} in sperm cells. Moreover, the concentration of intracellular Ca^{2+} eventually achieved after treatment with either drug alone or a combination of both drugs did not differ significantly. This suggested an increase in intracellular Ca^{2+} to be the main cause for the spermicidal effect.

The results of the study indicated benzamil to be a more potent spermicide than nonoxynol-9. Benzamil acted by elevating the intracellular Ca^{2+} in sperm cells. Hence, unlike nonoxynol-9, which produces vaginal lesions (Niruthisarad et al 1991; Roddy et al 1993), benzamil is envisaged to specifically target the sperm

cells. In addition, benzamil may be combined with other drugs that synergistically elevate intracellular Ca^{2+}, thereby resulting in a dose reduction of drugs for contact spermicidal action. However, further studies on systemic absorption and cytotoxic effects following vaginal application of benzamil are advocated before recommending its use for contraception.

References

Ashraf, M., Peterson, R. N., Russel, L. D. (1982) Activity and location of cation-dependent ATPase on the plasma membrane of boar spermatozoa. *Biochem. Biophys. Res. Commun.* **107**: 1273–1278

Bradley, M. P., Forrester, I. T. (1980) A sodium–calcium exchange mechanism in plasma membrane vesicles isolated from ram sperm flagella. *FEBS Lett.* **121**: 15–18

Breitbart, H., Darshan, R., Rubinstein, S. (1984) Evidence for the presence of ATP-dependent calcium pump and ATPase activity in bull sperm head membranes. *Biochem. Biophys. Res. Commun.* **122**: 479–484

Deshpande, S. B., Fukuda, A., Nishino, H. (1997) 3-Nitropropionic acid increases intracellular Ca^{2+} in cultured astrocytes by reverse operation of Na^{+}–Ca^{2+} exchanger. *Exp. Neurol.* **145**: 38–45

Edelstein, M. C., Fulghan, D. L., Gretly, J. E., Alexander, N. J., Bauer, T. J., Archer, D. F. (1991) Studies on the in vitro spermicidal activity of synthetic magainins. *Fertil. Steril.* **55**: 647–649

Hong, C. Y., Huang, J. J., Chiang, B. N., Wei, Y. H. (1986) The inhibitory effect of some ionophores on human sperm motility. *Contraception* **33**: 301–306

Niruthisarad, S., Reddy, R. F., Chutivangse, S. (1991) The effects of frequent N-9 use on the vaginal and cervical mucosa. *Sex. Transm. Dis.* **18**: 176–177

Reddy, K. V., Sahani, K. S., Meherji, K. P. (1996) Spermicidal activity of magainins: In vitro and in vivo studies. *Contraception* **53**: 205–210

Reynolds, T. R., Narang, B. S. (1984) In: Cheesebrough, M. (ed.) *Medical Laboratory Manual for Tropical Countries*, Vol. II. Butterworth and Co. Ltd, Kent, pp 186–187

Roddy, R., Cordero, M., Cordero, C., Fortney, J. A. (1993) A dosing study of nonoxynol-9 and genital discomfort. *Int. J. STD AIDS* **4**: 1165–1170

Siegel, P. K. S., Cragoe, E. J., Trumbie, M. J., Kaczorowski, J. G. (1984) Inhibition of Na^{+}/Ca^{2+} exchange in membrane vesicle and papillary muscle preparations from guinea pig heart by analogs of amiloride. *Proc. Natl. Acad. Sci. USA* **81**: 3238–3242

White, R. D., Jane, S. C., Ratnasooriya, W. D., Aitken, J. (1995) Complementary effects of propranolol and nonoxynol-9 upon human sperm motility. *Contraception* **52**: 241–247

WHO laboratory manual for examination of human semen and sperm–cervical mucus interaction (1999). Cambridge University Press, Cambridge