

Tocolytic effect of calcium channel blockers on buffalo uterus

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ABSTRACT

A study was conducted to evaluate the tocolytic effect of selective calcium channel blockers on uterine strips of buffaloes. Prostaglandin F₂ alpha (PGF₂α), oxytocin, (OT), potassium chloride (CK1) and barium chloride (BaCl₂) were used to induce uterine contractions of pregnant and diestrous stage buffaloes. After contracting the tissues, cumulative concentration-response curves of verapamil and diltiazem were constructed and their mean effective concentration (EC₅₀) values were determined and statistically compared. Comparative EC₅₀ values of CCBs revealed the rank order potency of PGF₂α > KC1 > BaCl₂ > OT on pregnant uteri while OT > PGF₂α > KC1 > BaCl₂ on dioestrus animals uteri for verapamil. The rank order potency of diltiazem was different from that of verapamil and it was KC1 > OT > PGF₂α > BaCl₂ on pregnant while KC1 > PGF₂α > BaCl₂ > OT on dioestrus stage uteri. Difference in the rank order potency of verapamil and diltiazem against different spasmogens on the uteri of pregnant or dioestrus stage buffaloes may be attributed to the difference in chemical structure of verapamil and diltiazem or their binding with calcium channels or due to difference in the molecular mechanism of different spasmogens-induced contraction. Further, it may not be unreasonable to state that CCBs appear to be promising tocolytics for buffaloes, however, detailed *in-vivo* pharmacological studies are warranted to establish their clinical utility and safety in farm animals including buffaloes.

Key words: Buffaloes, Calcium channel blockers, Diltiazem, Tocolytics, Verapamil

Free calcium ions (Ca²⁺) in sarcoplasm regulate the contractile activity of smooth muscles. An increase in intracellular free Ca²⁺ triggers the myometrial contraction (Anwer and Sanborn 1989). Tension development in uterus is largely dependent on extra-cellular Ca²⁺ rather than on intracellular Ca²⁺ pools. It is very well established that Ca²⁺ from the extra-cellular fluid via voltage-dependent calcium channels is an important step in the initiation of uterine smooth muscles contractility. Spontaneous and potassium chloride-induced tension development in rat uterus involves Ca²⁺ influx which is inhibited by CCBs (Granger *et al.* 1986, Longo *et al.* 2003). Different types of potassium channels including BK_{Ca} have also been reported to regulate myometrial contractility and relaxation mechanisms in experimental animals and also farm animals (Anwer *et al.* 1992, Toro *et al.* 1990, Mandi *et al.* 2005, Choudhury *et al.* 2009) Therefore, calcium channels blockers have been found

useful as probes for elucidating the function of Ca²⁺ channels and their quantification in excitable cells (Bolger *et al.* 1983, Janis and Triggle 1984).

Calcium channel blockers are potent smooth muscle relaxants and are widely used clinically for the treatment of cardiovascular diseases (Rang *et al.* 2003). Over the years, use of CCBs as tocolytic agents has also been suggested in the management of pre-term labour, threatened abortion and dysmenorrhoea (Rang *et al.* 2003). These have also been found to delay delivery in monkeys (Ducsay *et al.* 1987) and prevent or delay parturition in rats (Sharma and Gupta 1994). However, pharmacodynamics of CCBs-induced tocolysis of buffalo myometrium is yet to be elucidated. Therefore, present investigation was undertaken to study the tocolytic effects of selective CCBs on isolated uterine strips of pregnant and dioestrus stage buffaloes pre-contracted with different spasmogens.

MATERIALS AND METHODS

The uteri of non-descript breed buffaloes along with their ovaries were collected in chilled Ringer-Locke solution from the local abattoir of Mathura. Immediately after collection, material was brought to laboratory. Uterus, ovaries and corpus luteum on the ovary of ipsilateral side were thoroughly

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examined to determine the stage of estrous cycle or pregnancy. The stage of pregnancy was determined following the formula proposed by Soliman (Soliman 1975) by measuring the curved crown versus rump length. To study the tocolytic effect of CCBs, only the uteri of the dioestrus stage and pregnant buffaloes were used.

From the mid-cornual region of uterus, longitudinally a small piece of uterine tissue was collected; endo- and myometrial layers were gently removed and perimetrial strips of about 0.5×3.0 cm were prepared. Uterine strips were mounted in an organ bath containing 10 ml continuously aerated (with air) Ringer-Locke solution (pH 7.4) maintained at $37 \pm 0.5^\circ\text{C}$. The tissues were allowed to equilibrate under a constant tension of 2 gram for more than 1 hour. During equilibration period, the perfusion fluid was changed every 10 min. Spontaneous contractions of tissue(s) and their response(s) to different agonists/antagonists were recorded on a kymograph assembly using an isotonic frontal writing lever (magnification $\times 10$).

Prostaglandin $\text{F}_{2\alpha}$ ($0.8 \mu\text{M}$), oxytocin (0.004 IU/ml), potassium chloride (30 mM) and barium chloride ($200 \mu\text{M}$) were used to induce sustained myometrial contractions. Cumulative concentration response curves of calcium channel blockers, viz. verapamil and diltiazem, were constructed on spasmogen's pre-contracted uterine strips. Between 2 successive recordings of the concentration response-curves of CCBs, a minimum time period of 1.5 h was allowed and the perfusion fluid was changed every 10 min.

The cumulative concentration response curves (per cent of the maximum relaxation) of calcium channel antagonists (verapamil and diltiazem) against different spasmogens were plotted. The median effective concentrations (EC_{50}) to produce 50% relaxation for each CCB against different spasmogens(s) was calculated from the cumulative-concentration response curves and 95% confidence limits of ES_{50} were determined. The data were subjected to statistical analysis to determine the significant differences (Snedecor and Cochran 1967).

RESULTS AND DISCUSSION

Effect of verapamil and diltiazem on $\text{PGF}_{2\alpha}$ pre-contracted uterine tissues: Verapamil and diltiazem relaxed the $\text{PGF}_{2\alpha}$ ($0.8 \mu\text{M}$) precontracted perimetrial uterine strips of both the pregnant and dioestrus stage buffaloes in a concentration-dependent manner. The cumulative concentration response curves exhibiting the minimum threshold concentration (MTC) and the concentrations required to induce the maximal relaxant effect are shown in Fig. 1A. The EC_{50} values along with the confidence limits of verapamil and diltiazem required to relax the $\text{PGF}_{2\alpha}$ pre-contracted uterine strips are presented in Table 1.

Effect of verapamil and diltiazem on oxytocin (OT) pre-contracted uterine tissues: Both verapamil and diltiazem

Table 1. EC_{50} values of verapamil and diltiazem (molar) against different spasmogens on the isolated uterine strips of pregnant and dioestrus buffaloes ($n=5-9$)

Spasmogens (concentration in bath fluid/ml)	EC_{50} value of verapamil (M)		EC_{50} value of diltiazem (M)	
	Pregnant	Dioestrus	Pregnant	Dioestrus
$\text{PGF}_{2\alpha}$ ($0.8 \mu\text{M}$)	$1.83 \times 10^{-12\text{Aa}}$ (1.37×10^{-12} 2.45×10^{-12})	$1.20 \times 10^{-12\text{Aa}}$ (8.11×10^{-13} 1.77×10^{-12})	$8.39 \times 10^{-7\text{Aa}}$ (5.40×10^{-7} 1.30×10^{-6})	$8.18 \times 10^{-7\text{Aa}}$ (5.52×10^{-7} 1.22×10^{-6})
Oxytocin (0.004 IU/ml)	$1.21 \times 10^{-10\text{Ac}}$ (5.97×10^{-11} 2.44×10^{-10})	$2.45 \times 10^{-13\text{Bb}}$ (1.47×10^{-13} 4.08×10^{-13})	$1.43 \times 10^{-7\text{Ab}}$ (1.01×10^{-7} 2.01×10^{-7})	$1.27 \times 10^{-3\text{Bb}}$ (1.19×10^{-3} 1.35×10^{-3})
KCl (30 mM)	$2.00 \times 10^{-12\text{Aab}}$ (1.36×10^{-12} 2.94×10^{-12})	$3.63 \times 10^{-12\text{Ab}}$ (2.21×10^{-12} 5.94×10^{-12})	$4.78 \times 10^{-12\text{Ac}}$ (2.94×10^{-12} 7.78×10^{-12})	$6.22 \times 10^{-12\text{Ac}}$ (4.39×10^{-12} 8.82×10^{-12})
BaCl_2 ($200 \mu\text{M}$)	$3.54 \times 10^{-12\text{Ab}}$ (1.90×10^{-12} 6.58×10^{-12})	$1.75 \times 10^{-11\text{Bc}}$ (8.99×10^{-12} 3.42×10^{-11})	$1.28 \times 10^{-6\text{Aa}}$ (1.07×10^{-6} 1.53×10^{-6})	$8.59 \times 10^{-7\text{Ba}}$ (6.71×10^{-7} 1.10×10^{-6})

Data in parentheses indicates the confidence limits of the EC_{50} values; means in a row with different capital superscripts differed significantly; means in a column with different small superscripts differed significantly.

relaxed the OT (0.004 IU/ml) precontracted isolated uterine strips of pregnant and dioestrus stage buffaloes in a concentration-dependent manner. The MTC and the concentrations required to induce the maximal relaxant effect along with the cumulative-concentration response curves are shown in Fig. 1B. Table 1 depicts the EC_{50} values of verapamil and diltiazem along with their confidence limits.

Effect of verapamil and diltiazem on potassium chloride (KCl) pre-contracted uterine tissues: Verapamil and diltiazem relaxed the KCl (30 mM)-precontracted uterine strips of both the pregnant and dioestrus stage buffaloes in a concentration-dependent manner. The MTC and the concentrations required to induce the maximal relaxant effect along with the cumulative concentration response curves are shown in Fig. 2A. The EC_{50} values of verapamil and diltiazem along with their confidence limits are presented in Table 1.

Effect of verapamil and diltiazem on barium chloride (BaCl_2) pre-contracted uterine tissues: Barium chloride pre-contracted uterine tissues from both the dioestrus and pregnant buffaloes were effectively relaxed in a concentration-dependent manner by verapamil and diltiazem. The cumulative-concentration response curves of the CCBs under investigation showing MTC and the concentrations required for inducing maximal relaxation are shown in Fig. 2B. The EC_{50} values of verapamil and diltiazem along with their confidence limits are presented in Table 1.

Comparative assessment of the tocolytic efficacy of verapamil and diltiazem against different spasmogens: Based

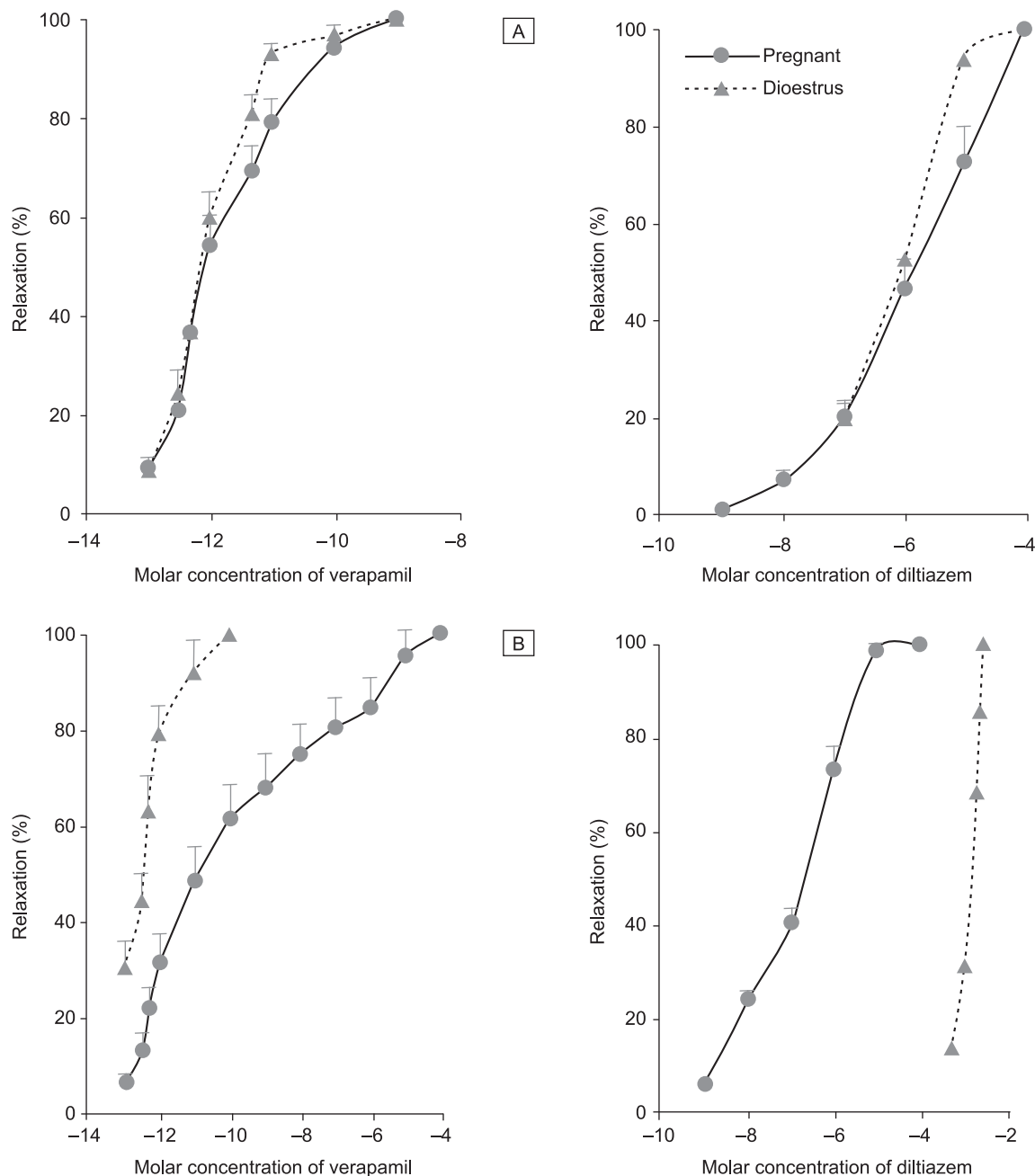


Fig. 1. Cumulative concentration response curves of verapamil and diltiazem against (A) $\text{PGF}_2\alpha$ (0.8 μM) and (B) oxytocin (0.004 IU/ml) precontracted isolated uterine strips of pregnant and dioestrus buffaloes (Vertical bars denote the SE of mean values (n=5-9)).

on the comparative EC_{50} values and the rank order potency of verapamil and diltiazem against different spasmogens on the uteri of pregnant and dioestrus stage buffaloes, it was evident that the uteri of pregnant animals precontracted with $\text{PGF}_2\alpha$ were most sensitive to the relaxant effect of verapamil with the rank order potency of $\text{PGF}_2\alpha > \text{KCl} > \text{BaCl}_2 > \text{OT}$ while oxytocin precontracted uterine tissues were more sensitive to diltiazem with the rank order potency of $\text{KCl} > \text{OT} > \text{PGF}_2\alpha > \text{BaCl}_2$.

Pharmacological response of dioestrus stage uteri pre-

contracted with different spasmogens was quite different from the uteri of pregnant animals. Unlike the maximal responsiveness of $\text{PGF}_2\alpha$ pre-contracted uterine strips of pregnant buffaloes, the dioestrus stage uteri precontracted with OT were most sensitive to the relaxant effect of verapamil as the rank order potency was $\text{OT} > \text{PGF}_2\alpha > \text{KCl} > \text{BaCl}_2$. Contrary to this, $\text{PGF}_2\alpha$ -precontracted uterine strips were more responsive to diltiazem and its rank order potency was $\text{KCl} > \text{PGF}_2\alpha > \text{BaCl}_2 > \text{OT}$.

Leaving aside the endogenously involved uterine

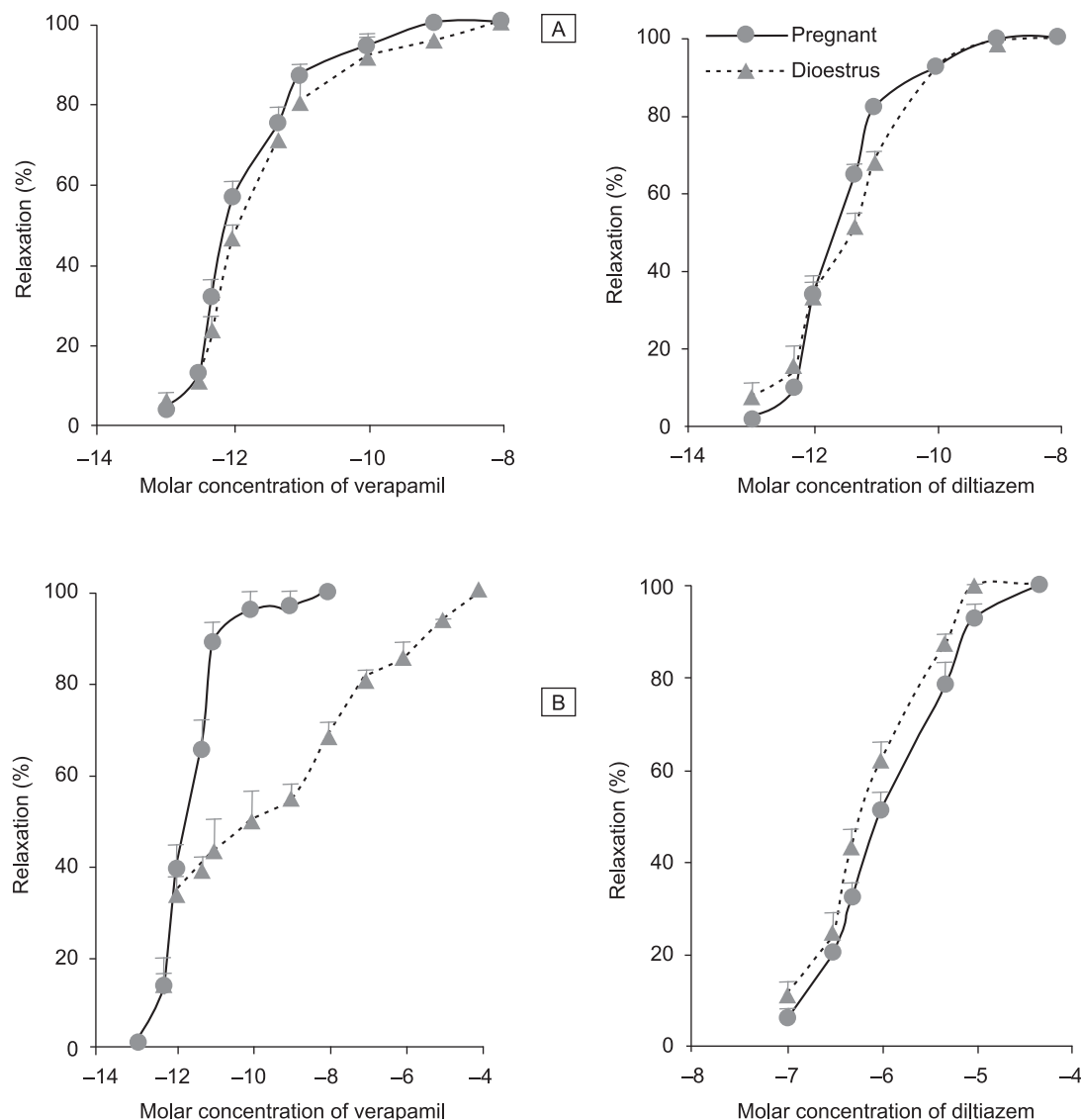


Fig. 2. Cumulative concentration response curves of verapamil and diltiazem against (A) potassium chloride (0.30m M) and (B) barium chloride (200 µm) precontracted isolated uterine strips of pregnant and dioestrus buffaloes (Vertical bars denote the SE of mean values (n=5-9)).

stimulants ($\text{PGF}_{2\alpha}$, OT), irrespective of the state of uterus, gravid or not, KCl-depolarized uterine tissues, compared to BaCl_2 , were more responsive to the tocolytic effect of both the CCBs. Further, specifically the KCl-depolarized uterine tissues (gravid or non-gravid) were found to be even more sensitive to the relaxant effect of diltiazem compared to the uterine strips precontracted with OT or $\text{PGF}_{2\alpha}$.

Statistical comparison of the EC_{50} values of verapamil and diltiazem against different spasmogens (Table 1) revealed that the dioestrus uterine strips precontracted with $\text{PGF}_{2\alpha}$ or OT were either equi- or significantly more sensitive compared to the uteri of pregnant animals to the tocolytic effect of both the CCBs used, except in case of diltiazem where OT pre-contracted pregnant uterine strips were more sensitive. Further, based on the comparative ranking and

statistical analysis of EC_{50} values, irrespective of the state of uterus or the spasmogens used, verapamil was found to be more potent than diltiazem.

Results of present study on isolated perimetrial strips from dioestrus and pregnant buffaloes demonstrated that CCBs, viz. verapamil and diltiazem, effectively relaxed the $\text{PGF}_{2\alpha}$, OT, KCl or BaCl_2 pre-contracted tissues in a concentration-dependent manner. These produced prolonged uterine relaxation, which not only differed with the state of uterus (gravid or dioestrus) but also with the spasmogens; thus suggesting that both, verapamil and diltiazem, are promising tocolytics, for buffaloes as has been reported in other species of animals (Abel and Hollingsworth 1985, Lirette *et al.* 1985, Calixto and Antonio 1986, Poli *et al.* 1987, Janis and Triggler 1984).

Based on the comparative EC_{50} values of verapamil against different spasmogens, the rank order potency of verapamil on pregnant uteri was $PGF_{2\alpha} > KCl > BaCl_2 > OT$ while on the dioestrus uteri, it was $OT > PGF_{2\alpha} > KCl > BaCl_2$. Further, the uteri of pregnant animals precontracted with $PGF_{2\alpha}$ were most sensitive to the relaxant effect of verapamil while least when precontracted with oxytocin. However, the response was just reverse in case of dioestrus stage uterus. The rank order potency of diltiazem on pregnant uterine tissue was $KCl > OT > PGF_{2\alpha} > BaCl_2$ while on the dioestrus uteri, it was $KCl > PGF_{2\alpha} > BaCl_2 > OT$. The overall comparison of the EC_{50} values of verapamil and diltiazem against different spasmogens on the pregnant and dioestrus uteri revealed that irrespective of the state of uterus or the spasmogens used ($PGF_{2\alpha}$, OT, KCl, $BaCl_2$), verapamil was found to be more potent than diltiazem.

The difference in rank order potency of verapamil and diltiazem against different spasmogens may be attributed to difference in their chemical structures as these belong to two different subclasses of CCBs (Catterall and Striessnig, 1992) or/and the difference in mechanism of action/binding of verapamil and diltiazem to their respective sites (Triggle and Swamy 1983). Variations in tocolytic potency and efficacy of CCBs against different spasmogens has earlier also been reported (Coruzzi *et al.* 1989a, b, Sharma and Gupta 1993).

Studies further revealed that the dioestrus stage uterine strips precontracted with intrinsically involved and conventionally used myometrial stimulants, viz. $PGF_{2\alpha}$ and oxytocin, were equally or/and significantly ($P < 0.05-0.001$) more sensitive to the tocolytic effect of verapamil and diltiazem. Oxytocin inhibits the uptake of Ca^{2+} into bovine myometrial sarcoplasmic reticulum (Carstens 1974) while $PGF_{2\alpha}$ acts by liberation Ca^{2+} from intracellular binding sites and influx of extracellular Ca^{2+} (Sharma and Gupta 1993, Liggins 1979). Potassium chloride-induced depolarization opens voltage-operated calcium channels in plasma membrane of cells, thus allowing the extracellular Ca^{2+} entry into cells while $BaCl_2$ directly stimulates the muscles probably through Na^+ or their ion channels (Sollmann 1957). From this, it is evident that the molecular sites/mechanism of action of different spasmogens are different, therefore, differences in tocolytic efficacy/potency of verapamil and diltiazem on the uteri of pregnant or dioestrus animals may involve some/different subtle cellular or molecular mechanisms. Female sex hormones are known to regulate uterine calcium channels (Ischii *et al.* 1986, Batra 1987, Ruzycky *et al.* 1987) but endocrinologically, the uteri of pregnant (1–5 months) and dioestrus animals are almost similar and estrogen and progesterone levels and their ratio does not differ significantly (Arora and Pandey 1982), therefore, the possibility of differences in tocolytic efficacy of CCBs due of some extra-hormonal factors/mechanisms can not be ruled out.

Further KCl-depolarized tissues required least

concentrations of diltiazem or verapamil to induce uterine relaxation compared to barium chloride irrespective of the state of uterus whether gravid or nongravid. Thus, indirectly indicating the possibility of CCBs-induced augmentation of outward K^+ current through opening of some K^+ channels (K_v , BK_{Ca} , K_{ATP}). Compared to the present studies with CCBs, KCl-depolarized uterine tissues of buffaloes required highest concentrations of selective β_2 adrenergic agonists for inducing tocolysis (Garg *et al.* 2004). Recently, existence of functional BK_{Ca} , K_{ATP} , channels has been documented by this Department in buffalo myometrium (Choudhury 2007, Choudhury *et al.* 2009).

Therapeutic use of CCBs for the management of pre-term labor, threatened abortion and dysmenorrhoea is well established. Present *in-vitro* studies also indicate good therapeutic potential of CCBs as tocolytics in buffaloes; however, detailed *in-vivo* pharmacological studies are warranted to establish their clinical utility and safety in farm animals including buffaloes.

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