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Augmentation of capsaicin-induced vasorelaxation of superior mesenteric artery (*Capra hircus*) in acidosis: role of TRPV1 channels

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Capsaicin is known to induce vasorelaxation through activation of the transient receptor potential cation channel subfamily V member 1(TRPV1). However, TRPV1 is a nonselective cation channel and can be activated by a wide variety of exogenous and endogenous stimuli, including acidic conditions. The present study investigated the effect of changes in *p*H on capsaicin-induced vasorelaxation in both endothelium intact and denuded goat superior mesenteric artery (GSMA) rings, using a highly sensitive isometric force transducer. There was a significant increase in capsaicin-induced vasorelaxation in nor adrenaline (NA)-precontracted GSMA rings at acidic *p*Hs of 6.8 (44.71±4.85%) and *p*H 6.0 (46.43±2.59%), compared to a physiological level of *p*H of 7.4 (33.25±2.62%). Although not completely abrogated, endothelium denudation and ruthenium red (RR) treatment significantly attenuated capsaicin-induced vasorelaxation at all *p*H levels. These data together suggest that, while other minor endothelium/TRVP1-independent mechanisms may exist, augmentation of capsaicin-induced vasorelaxation in acidosis is primarily endothelium-dependent and mediated through TRPV1 channels.

Keywords: Acidosis, Endothelial TRPV1, Capsaicin, Goat superior mesenteric artery

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Capsaicin, (8-methyl-N-vanillyl-trans-6-nonenamide) (Fig. 1) the main pungent ingredient in hot chili peppers has been used since time immemorial around the globe as a spice for enhancing the flavor, as defense by temporarily blinding eyes (pepper spray) and as an analgesic[1]. Fig. 1 represents the chemical structure of capsaicin. It is the main capsaicinoid in chili peppers, followed by dihydrocapsaicin and other minor capsaicinoids nordihydrocapsaicin, homodihydro capsaicin, and homocapsaicin[2]. Capsaicin plays an important role in maintenance of physiological homeostasis by increasing thermogenesis, enhancing catecholamine secretion from adrenal medulla, decreasing weight gain, and adipogenesis by enhancing energy metabolism[3,4]. Capsaicin is a highly selective agonist for the transient receptor potential vanilloid 1 (TRPV1) cation channel[5]. Transient receptor potential vanilloid receptor 1 (TRPV1) is a polymodal nociceptor model of the TRPV subfamily which can be activated by capsaicin, noxious heat, extracellular protons, vanilloids or voltage dependent depolarization[5]. It is predominately expressed in the small diameter afferent neurons[6] and also in non-neuronal tissues like blood vessels[7-9]. The main functions in different organ systems include pain perception, attenuation of inflammatory pain conditions and modulation of the receptor activity[10].

TRPV1 is distributed along the vascular system and is involved in regulation of normal vascular smooth muscle tone, resulting in either vasoconstriction or vasodilatation, depending on the physiological condition[4,11]. TRPV1 activation results in the release of substance P, a neurokinin, which binds to neurokinin 1 (NK1) receptor resulting in vasoconstriction[12-14]. Vasodilatory effects result from both neuronal and vascular mechanisms of TRPV1

Fig. 1- Chemical structure of capsaicin (Reyes-Escogido *et al.,* 2011)

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activation in different vascular bed[4,11,15-17]. Capsaicin has also been reported to cause vasorelaxation in human coronary artery, rat aorta[18], guinea pig ileum[19], human and porcine coronary arteries[20] and rat mesenteric artery[4,17].

The mesenteric arterial bed supplies blood to major portion of splanchnic vascular bed[21] and regulates blood pressure[22,23]. Hence, goat mesenteric artery could be considered as a model for study of vasomotor changes under the influence of acidosis at molecular and functional level. Earlier morphological study showed that only 32% of vagal afferents supplying the mouse jejunum contain TRPV1-positive fibres[24]. TRPV1 expressed by primary sensory neurons in the digestive tract are involved in the modulation of gut sensitivity and play an important role in inflammation and pain condition[25]. The pathological conditions like inflammation, ischemia, and infections result in elevated proton concentrations and fall in *p*H ≤ 6 in the surrounding vascular bed[26]. One consequence of tissue acidification is the stimulation of sensory nerves via the polymodal H^+ gated trans-membrane channels TRPV1 receptor[27]. Acidic *p*H has been shown to stimulate a subpopulation of sensory nerves that are also activated by capsaicin, however the underlying mechanism for both TRPV1 agonist capsaicin and high H^+ is different, as reported by site-directed mutagenesis studies[28]. An increased expression level for TRPV1 receptors in inflammatory condition also has been detected in human gut[25,29]. Till date, to the best of our knowledge no information is available on the presence of TRPV1 channels in goat mesenteric artery. Morever, the role of extracellular acidosis in mesenteric artery in *Capra hircus* Linnaeus, 1758 is also unexplored. Therefore, in the present study, we tried to characterize capsaicin-induced vasorelaxation under physiological environment (*p*H 7.4) and also explore the modulator effect of acidosis (*p*H 6.8 or 6.0) on TRPV1 activation by capsaicin in goat superior mesenteric artery.

Materials and methods

Preparation of superior mesenteric artery and functional study

After careful exposure of goat intestinal mesentery a branch of superior mesenteric artery adjacent to the duodenum and jejunum just before its branching into inferior branch was dissected out and placed in cold aerated modified Krebs-Henseleit saline (MKHS) solution (mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 11.9, KH2PO4 1.2 and Dextrose 11.1 (*p*H 7.4). The solution was adjusted to either *p*H, 7.4, 6.8 or 6.0 by using 1N HCl. Arteries were cleared of fat and connective tissues. Endothelium was removed by cotton swab method. The arterial ring of 1.5-2 mm were then mounted between two fine stainless steel L-shaped hooks and kept under a resting tension of 1.5 gm in a thermostatically controlled (37.0±0.5 ºC) automatic organ bath (Pan Lab) of 20 mL capacity containing MKHS and was aerated continuously with air. The arterial rings were equilibrated for 90 min before recording of muscle tension. During this period the bathing fluid was changed every 15 min. This experiment was repeated for both endothelium intact and denuded vessels. The change of isometric tension was measured by a highly sensitive isometric force transducer (Model: MLT0201, AD instrument, Australia) and analysed using chart 7.1.3 software.

Experimental protocol

Effect of *p*H (7.4 or 6.8 or 6.0) on Capsaicin (1ηM to 10μM)-induced vasorelaxation in NA (10μM)-pre-contracted GSMA rings in absence or presence of endothelium or Ruthenium Red (10μM).

In order to examine the sensitivity of TRPV channels under acidic stress, capsaicin induced (1ηM to 10μM)-induced vasorelaxation in NA (10μM)-pre-contracted GSMA rings was elicited by cumulative addition of capsaicin at an interval of 4 min in both endothelium intact (ED+) or denuded (ED-) rings. In order to examine sensitivity of TRPV1, the arterial rings were pre incubated with Ruthenium Red (10μM) for a period of 30 min prior to NA-induced contraction. The concentration-related contractile response curves (CRCs) of capsaicin were elicited and shift of the CRCs were compared with non treated control. The CRC response curves of Capsaicin induced relaxation in absence of endothelium or presence of Ruthenium Red was plotted and -LogEC_{50} were calculated. The -LogEC_{50} and shift of the CRC response curves were compared for each set of experiments. Similarly, mean pD_2' were compared to evaluate the blocking effect of antagonists under different *p*H ranges.

Statistical analysis

E_{max} and E_{Bmax} are the maximal contraction heights in the absence and in the presence of the antagonist respectively. The data was expressed as percentage of the maximum response to agonist obtained in the

absence of antagonist (control) and analyzed by the interactive non-linear regression through the computer program Graph Pad Prism (Graph Pad Prism Software, San Diego, CA, USA). E_{max}/E_{Bmax}, mean threshold concentration and $-LogEC_{50}/EC_{50}$ were calculated through Graph Pad Prism. Graph Pad Quick Calcs 't' test was used to calculate the P value to determine the level of significance and to analyze the data. A 'p' value < 0.05 was considered statistically significant.

Drugs

Nor adrenaline (Merck, India); Ruthenium Red (MP Biochemicals), Capsaicin (Sigma, USA) was the drugs employed for isometric contraction study. All the solutions were prepared fresh in triple distilled water except Capsaicin which is soluble in 70% ethanol.

Results

Capsaicin-induced vasorelaxation in NA (10µM)-induced contraction at *p*H 7.4, 6.8 and 6.0.

Capsaicin (1ηM to 100µM)–induced concentration dependent vasorelaxation was significantly increased at *p*H 6.8 or 6.0 as compared to control *p*H 7.4. The concentration related vasorelaxation curves of capsaicin at *p*H 7.4 (E_{max} 33.25±2.62%; pD_2 7.94±0.26, n=11) was shifted to left at *p*H 6.8 with a significant increase in E_{max} and pD_2 (44.71±4.85%; 8.26±0.41, n=9), and also at *p*H 6.0 with a significant increase in E_{max} (46.43±2.59%) and non significant decrease in pD_2 (7.78±0.17, n=14) (Fig. 2; 2A). Table 1 compares the E_{max}/E_{Bmax} and pD_2 value for capsaicin induced vasorelaxation at *p*H 7.4, 6.8 and 6.0 in

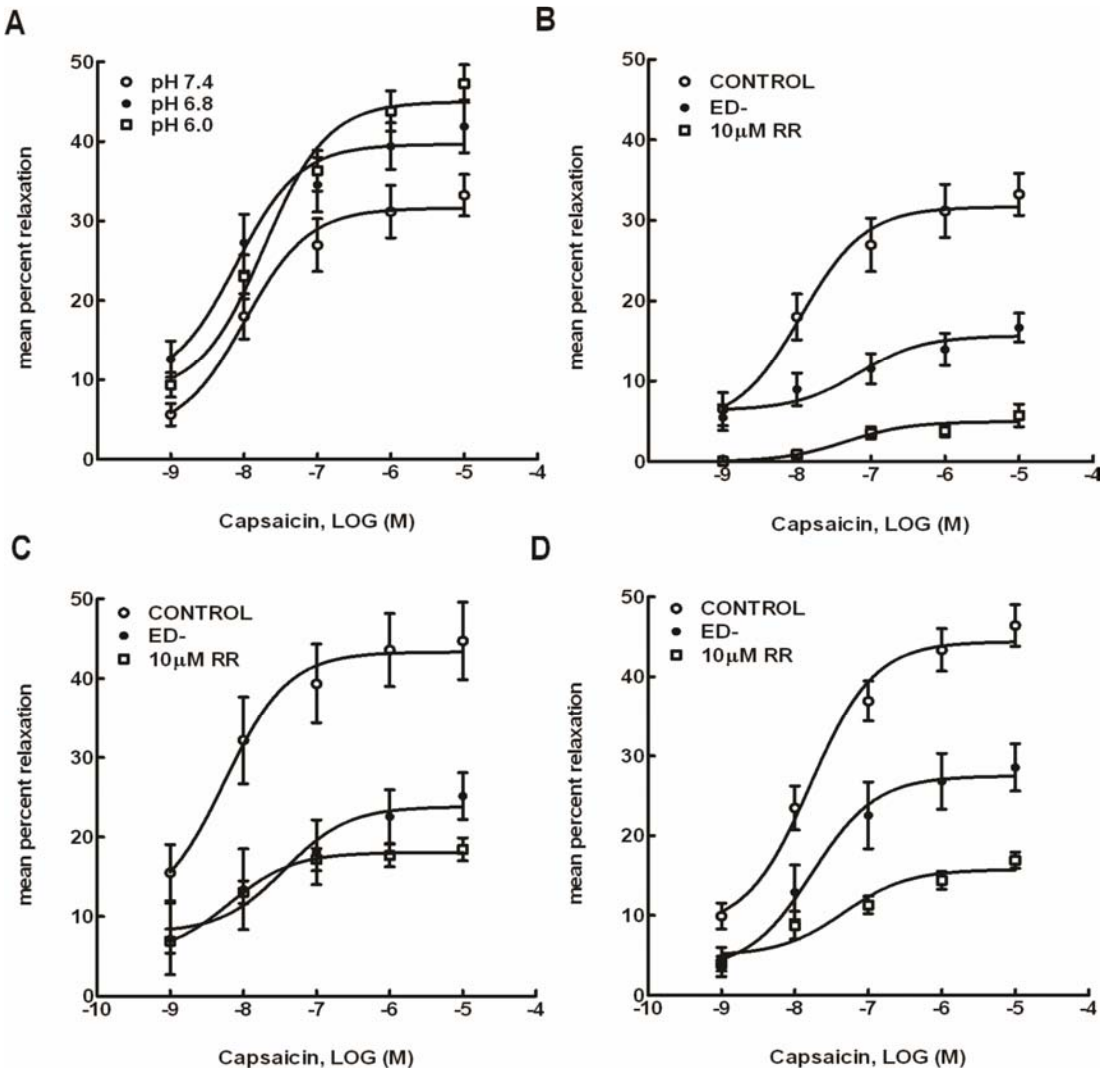

Fig. 2 - Capsaicin (1ηM-10µM)-induced concentration related vasorelaxation in NA(10µM) precontracted isolated goat superior mesenteric arterial rings at (A) *p*H 7.4, 6.8 or 6.0 and in absence or presence of endothelium or Ruthenium Red (10µM) at (B) *p*H7.4, (C) *p*H 6.8 (C) *p*H 6.0.

Table 1-Capsaicin (1ηM-10μM)-induced vasorelaxation on NA (10μM) precontracted isolated GSMA rings in presence or absence of endothelium and Ruthenium Red (10μM) at *p*H 7.4 or 6.8 or 6.0.

	E_{max}/E_{Bmax}			pD_2		
	*p*H 7.4	*p*H 6.8	*p*H 6.0	*p*H 7.4	*p*H 6.8	*p*H 6.0
Capsaicin	33.25±2.62 (n=11)	44.71±4.85[a] (n=9)	46.43±2.59[a] (n=14)	7.94±0.26	8.26±0.41	7.78±0.17
ED-	16.70±1.79[a] (n=8)	25.16±2.49 [b] (n=6)	28.58±2.96[b] (n=7)	7.16±0.45	7.44±0.62	7.76±0.33
10μM RR	5.74±1.39 [a] (n=8)	18.46±1.45 [b] (n=6)	16.94±1.02[b] (n=7)	7.34±0.38	8.19±0.3	7.33±0.28

[a](p<0.05) represents level of significance between the columns within each row. Data of control row (non-treated) were compared with the data of *p*H 7.4 between the columns. [b](p<0.05) represents level of significance between rows within each column. Data of each row (treated) in a particular column is compared with the corresponding control (non-treated). E_{max} and pD_2 values are expressed as mean±SEM, n= no of experiments.

GSMA rings. In conclusion, capsaicin induced vasorelaxation in NA-precontracted ED+ GSMA rings was augmented with decrease in *p*H.

Capsaicin induced vasorelaxation in NA (10μM)-precontracted GSMA rings in absence or presence of endothelium and 10 μM RR at *p*H 7.4, 6.8 and 6.0.

Effect of endothelium denudation and non specific TRPV1 blocker, RR (10μM) on capsaicin induced vasorelaxation at *p*H 7.4, 6.8 and 6.0 has been presented in Fig. 2B-D. At *p*H 7.4, in presence of endothelium denudation and 10μM RR, capsaicin induced vasorelaxation curve was shifted to right with significant (P<0.05) decrease in E_{Bmax} and non significant decrease in pD_2 (ED-: 16.70±1.79%, 7.16±0.45, n=8; RR: 5.74±1.39%; 7.34±0.38, n=8) as compared to non treated control (E_{max} 33.25±2.62%; pD_2 7.94±0.26, n=11) (Fig. 2B).

At *p*H 6.8, in presence of endothelium denudation and 10μM RR, capsaicin induced vasorelaxation curve was shifted to right with significant (P<0.05) decrease in E_{Bmax} and non significant decrease in pD_2 (ED-: 25.16±2.49%, 7.44±0.62, n=6; RR: 18.46±1.45%; 8.19±0.30, n=6) as compared to non treated control (E_{max} 44.71± 4.85%, pD_2 8.26±0.41, n=9) (Fig. 2C).

At *p*H 6.0, in presence of endothelium denudation and 10μM RR, capsaicin induced vasorelaxation curve was shifted to right with significant (P<0.05) decrease in E_{Bmax} and non significant decrease in pD_2 (ED-: 28.58±2.96%, 7.76±0.33, n=7; RR: 16.94±1.02%, 7.33±0.28, n=7) as compared to respective non treated control (E_{max} 46.43±2.59%; pD_2 7.78±0.17, n=14) (Fig. 2D). Table 1 compares the E_{max}/E_{Bmax} and pD_2 value for capsaicin induced vasorelaxation in absence or presence of endothelium and 10μM RR at *p*H 7.4, 6.8 and 6.0 in GSMA rings. In conclusion, endothelium denudation and RR inhibited capsaicin induced vasorelaxation by 17-20% and 26-29% at all *p*H, respectively.

Discussion

In the present study, we observed that, (i) capsaicin, a TRPV1 channel agonist caused vasorelaxation in a concentration dependent manner at physiological *p*H 7.4 (33%) which was augmented at reduced *p*H 6.8 (45%) and 6.0 (46%), (ii) endothelium denudation inhibited capsaicin induced vasorelaxation by 17-20% at all *p*H (iii) Ruthenium Red, a non-selective TRPV1 blocker attenuated capsaicin induced vasorelaxation at *p*H 7.4 (83%), *p*H 6.8(59%) and *p*H 6.0 (64%).

Capsaicin, a pungent constituent of red pepper, activates sensory C-fibres via transient receptor potential vanilloid receptors type 1 (TRPV1)[5,1]. The effect of capsaicin on vascular tone and blood pressure is a paradox[4]. TRPV1 channels activation caused indirect release of substance P, a neurokinin, which binds to neurokinin 1 (NK1) receptor resulting in vasoconstriction in rat coronary artery[12], rat mesenteric artery[13], and rat aorta[14]. Vasodilatory effects result from both neuronal and vascular mechanisms of TRPV1 activation[17,11] via release of Calcitonin Gene Related Peptide (CGRP)[30], stimulation of protein kinase A and epithelial nitric oxide synthases, which releases nitric oxide[4], or via TRPV1-independent modulation of COX-2 enzymatic activity[15], activation of Ca^{2+}-activated K^+ channels[22] and inhibition of L-Type Calcium Channels[16]. Earlier studies reported that capsaicin induced vasorelexation in human coronary artery with pD_2 5.27±0.12, porcine coronary artery with pD_2 5.27±0.09[22] and rat mesenteric artery with pD_2 6.88 ± 0.16[4]. In the present study, capsaicin (1ηM-10μM) resulted in concentration dependent vasorelaxation at physiological *p*H (7.4) with pD_2 7.94±0.26 suggesting that TRPV1 channel is expressed in GSMA and exhibited a greater sensitivity to capsaicin as compared to human coronary artery[22] and porcine coronary artery[22] and rat mesenteric artery[4]. Ruthenium red, a water soluble polycationic dye, was

found to block the pore of the capsaicin-operated cation channel TRPV1 thus interfering with all polymodal ways of TRPV1 activation[31]. This TRPV1 channel blocker has been reported to inhibit TRPV1 mediated vasorelaxation in human coronary artery at 0.1mM by 94%, rat skin saphenous nerve preparation at 50μM[31] and rat mesenteric artery at 3μM by about 40%[32]. 10 μM Ruthenium Red inhibited the capsaicin induced vasorelaxation by 83% at *p*H 7.4 indicating that in GSMA, TRPV1 channel possess antagonist binding sites that exhibited greater sensitivity to RR.

TRPV1 induced vasorelaxation may be mediated by release of relaxing factors via activation of endothelial TRPV1 channel, which mediates an increased Ca^{2+} influx and subsequent phosphorylation of PKA and eNOS, followed by release of cAMP and Nitric oxide[4]. We observed that endothelial denudation in GSMA rings reduced capsaicin induced vasorelexation by 16% out of 33%, suggesting that 50% of the vasorelaxation of capsaicin is endothelium dependent and 50% is endothelium independent. The endothelium dependent vasorelaxation could be mediated by activation of both PKA and eNOS[4]. Endothelium independent vasodilatory effects on activation of TRPV1 channels in GSMA could be mediated *via* release of neuropeptides from perivascular nerve fibres like Calcitonin Gene Related Peptide (CGRP)[29], neurokinin A and substance P[11,17] or TRPV1-independent modulation of COX-2 enzymatic activity[15], activation of Ca^{2+}-activated K^+ channels[22] and inhibition of L-Type Calcium Channels[16].

In order to examine the influence of acidic *p*H on TRPV1 function, we elicited capsaicin induced vasorelaxation at acidic *p*H and observed that capsaicin induced vasorelaxation was augmented with decrease in *p*H 7.4 (33%) → 6.8 (45%) → 6.0 (46%). Such augmentation of vasorelaxation could be due to (i) additive or synergistic effects of acid on TRPV1 activation by capsaicin[27] and (ii) amplification either endothelium dependent or endothelium independent mechanisms. In rat TRPV1 channels expressed in HEK-293, it has been demonstrated that acidic *p*H potentiated the capsacin binding to TRPV1 channels could be due to slowing of the deactivation rate and an increase in the activation rate of capsaicin-activated currents[27]. So, in our present study capsaicin induced vasorelaxation obtained at 1μM (31.18±3.3%) at physiological *p*H 7.4 was observed at 10ηM (32.19±5.49%) at *p*H 6.8 and at 50ηM (36.32±2.55%) at *p*H 6.0. Thus, under acidic conditions, about 100 fold increase in affinity for capsaicin at TRPV1 channels and this could be a reason for augmentation of vasorelaxation. A constant fraction of vasorelaxation (16-17%) was attenuated in ED- GSMA rings at *p*H 7.4, 6.8 and 6.0 suggested that capsaicin induced vasorelaxation under acidic *p*H doesn't directly activate the eNOs or via activation of COX enzyme which contradicts that TRPV1 mediated vasorelaxation is contributed by eNOs and is activated under acidosis[4]. In contrast, our findings suggest that there is increase in endothelium independent component of vasorelaxation under acidosis in GSMA rings could arise due to increase in binding sites which in turn activated neuronal mechanisms of TRPV1 activation[11,17] via release of Calcitonin Gene Related Peptide (CGRP)[30]. It could also be predicted that the increase in vasorelaxation by capsaicin in GSMA which may due to activation of Ca^{2+}-activated K^+ channels[22] and inhibition of L-Type Calcium Channels[16]. Since, TRPV1 channels induced vasorelaxation is augmented under acidic stimulation, it could be possible that there is altered potency/ efficacy of TRPV1 antagonist. We observed that blocking effect of 10μM RR at *p*H 7.4 (83%) was reduced to 50-60% under acidic *p*H 6.8 and 6.0. Under physiological *p*H, the binding of RR to antagonist binding site could undergo conformational changes leading to reduced affinity for polymodal binding to TRPV1 channels. Increased vasorelaxation by capsaicin, unaltered endothelial relaxing factors and decreased antagonistic effect of RR under acidosis induced activation of TRPV1 channels could be explained by possible mechanism arising from binding of protons that alter confirmation of TRPV1 channels to cause trapping of capsaicin, so it can't be released unless proton interaction is terminated[27]. The data of our study suggest that H^+ ion or increase in occupancy of the proton binding site of TRPV1 resulted in an allosteric change in the receptor that prevent the release of capsaicin.

Since, most of pathophysiological conditions like inflammation, ischemia or vascular shock are associated with tissue acidosis, the present study will contribute towards the study on role of TRPV1 channels in maintaining vascular tone under acidosis. The present result would enhance the understanding of the role of TRPV1 channels in gut pathophysiology and can be exploited as a possible target for therapy in tissue acidosis arising from disease states.

Traditional significance of study to the farmers/ researchers/country

Traditionally used worldwide chilli peppers, members of the genus *Capsicum*, gives the "hot" sensation due to presence of the alkaloid capsaicin. *Capsicum* has been known since the beginning of civilization and has been a part of the human diet since about 7500 BC. It was purified and named as capsaicin (Fig.1) in the 19th century and first synthesized in the 1920s. Beyond their widespread use as a spice, chilli peppers were used in the Americas by the Aztecs and Tarahumara Indians as a remedy for coughs and bronchitis. The genus Capsicum is a member of the Solanaceae family that includes tomato, potato, tobacco, and petunia. The genus Capsicum consists of approximately 22 wild species (*C. annuum, C. baccatum, C. chinense, C. frutescens,* and *C. pubescens*) and five domesticated species (Fig. 3). As a medicinal plant, Capsicum species has been used as a carminative, digestive irritant, stomachic (beneficial to gastric digestion), stimulant, rubefacient, and tonic. The plants have also been used as folk remedies for dropsy, colic, diarrhoea, asthma, arthritis, muscle cramps, and toothache. High levels of ground hot pepper have induced stomach ulcers and cirrhosis of the liver in laboratory animals. Capsicum peppers may stimulate body temperature, flow of saliva, and gastric juices. However, modern usage of capsaicin is focused on the treatment of various types of pain (see below) and also in the treatment of detrusor hyperreflexia, a form of urinary incontinence. High-dose oral capsaicin also has anticancer properties in some animal model studies but seems to be a cancer promoter in others. TRPV1, is a Ca2+ ion channel that also responds to, and integrates, signals from piperine (the irritant in black pepper), protons, and other noxious stimuli. In the field of nociceptor pharmacology capsaicin has been used to desensitize nociceptors and its antagonists have been used to block pain pathways. A progressive acidosis can induce vasodilatation leading to hypotension and vascular shock. In ruminants usually acidosis causes an exuberant economic loss to farmers due reduction of milk production. It is obvious that increased acidosis could cause increased function an expression of TRPV1 Channels which can be treated by using TRPV1 Channels antagonists. Our present study showed that vasorelaxation induced by capsaicin in GSMA is increased with increase in acidic *p*H and it is due to augmented function or expression of TRPV1 channels of the vascular smooth muscle cells. On the basis of scientific observation it may be recommended that addition of capsaicin or capsaicin containing fodder to ruminants could be useful in reducing metabolic acidosis. Similarly, identification of a natural antagonist of TRPV1 channels could be an appropriate therapy to treat acidosis in addition to other therapeutic strategy.

Fig. 3- *Capsicum annuum* (Cayenne)

Conflicts of interest

The authors indicate no potential conflicts of interest.

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